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Genetic Variability in *Saccharomyces cerevisiae* due to Osmotic Stress

by

Kenneth R. Parker ©

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA
Spring, 1993

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "GENETIC VARIABILITY DUE TO OSMOTIC STRESS" submitted by Kenneth R. Parker in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in the Department of Genetics.

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This thesis is dedicated to my mother and Colleen, and to the memory of my father for all the love and support that this close family provided.

ABSTRACT

Osmotic stress can be mutagenic and recombinagenic to *Saccharomyces cerevisiae*, depending upon the adaptive state of the cells to conditions of water deficit. Exposure of logarithmic phase cells to an abrupt increase in solute concentration constitutes an osmotic shock and is highly lethal, mutagenic and recombinagenic. Stationary phase cells are not affected. As the concentration of solutes is lowered, the response is reduced to a level which is antimutagenic, but which effectively demarcates a lower limit of osmolality or threshold for this mutagenic phenomenon. The mutagenic effects of osmotic shock are largely independent of solute chemistry, as indicated by NaCl, KCl, $MgCl_2$, $CaCl_2$, Na_2SO_4, glucose and sorbitol. Some solutes (saccharin salts and NH_4Cl) are more mutagenic than would be predicted from osmolality alone, which may be due to their effects on gene expression or possibly DNA reactivity, as influenced by activating enzymes. Osmotic shock in diploid strains of yeast induces interchromosomal recombination, especially crossing over. Frameshift mutations are produced immediately following an osmotic shock of 1.0 M NaCl, whereas base substitutions are induced as a delayed response, indicating that these two types of mutations arise by different mechanisms. Application of an osmotic shock without removing the growth medium is less mutagenic, especially for base substitutions. The growth medium contains glucose which allows the cells to assimilate the primary osmolytes (glycerol and trehalose) used by *S. cerevisiae* to counteract the osmotic loss of water. When yeast cells are induced to grow in a medium supplemented with 0.30 to 0.60 M NaCl, the spontaneous mutation rates are not significantly affected. However, the mutation spectrum is appreciably changed, as indicated by forward mutations in the *URA3* gene. All base substitutions to adenine (A) or thymine (T) and frameshift mutations are increased, but substitutions to guanine (G) or cytosine (C) are reduced when NaCl is present. There is a significant distributional bias among the base substitutions between the 5'- and 3'-ends of the gene. This mutational specificity can be explained in terms of mutagenic DNA repair enzymes, wherein *REV3* and *RAD18* represent at least two genes that are required for expression of this phenomenon. It is hypothesized that these

repair enzymes serve to balance the probability of occurrence of different types of mutation (e.g., base substitutions to A/T vs. G/C) in different regions of a gene (or the genome).

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LIST OF ABBREVIATIONS

A, dATP	Deoxyadenosine nucleotide
ade	Adenine
AP	Apurinic or abasic site
arg	Arginine
C, dCTP	Deoxycytidine nucleotide
cfu	Colony forming unit
C-terminal	Carboxyl-terminal portion of a protein, with reference to the corresponding region of the gene which encodes the 3'-portion of an mRNA
CTP	Cytidine 5'-triphosphate
Δ	Deletion of all or part of a gene
ddATP	2', 3'-Dideoxyadenosine 5'-triphosphate
ddCTP	2', 3'-Dideoxycytidine 5'-triphosphate
ddGTP	2', 3'-Dideoxyguanosine 5'-triphosphate
ddTTP	2', 3'-Dideoxythymidine 5'-triphosphate
DSB	Double-strand break
EDTA	Ethylenediaminetetraacetic acid
5-FOA, FOA	5-Fluoroorotic acid
FOA^R	Fluoroorotic acid-resistant
G, dGTP	Deoxyguanosine nucleotide
his	Histidine
hom	Homoserine
J/m^2	Joules per square metre
LB	Luria-Bertani medium
leu	Leucine
M	Molar
MC	Mortimer complete medium
µg	Microgram
µm	Micrometre
ml	Millilitre
MML	Method of maximum likelihood
mRNA	Messenger ribonucleic acid
NER	Nucleotide excision repair
nm	Nanometre

N-terminal	Amino-terminal portion of a protein, with reference to the corresponding region of the gene which encodes the 5'-portion of an mRNA
OMP	Orotidine-5'-monophosphate
ORF	Open reading frame
Os/kg	Osmolal per kilogram
PCI	70% phenol: 30% chloroform: 1% isoamyl alcohol
PCR	Polymerase chain reaction
SC	Synthetic complete medium
SOS	Regulon in prokaryotes which "rescues" the cell under stress conditions
SSB	Single-strand break
T, dTTP	Deoxythymidine nucleotide
TE	10mM Tris·HCl, 1mM EDTA (unless specified otherwise)
thr	Threonine
Tris	Tris (hydroxymethyl) aminomethane
tRNA	Transfer ribonucleic acid
trp	Tryptophan
ura	Uracil
UTP	Uridine 5'-triphosphate
uv	Ultraviolet
YEPD, YD	Yeast extract, peptone & dextrose medium
YNB	Yeast nitrogen base

CHAPTER 1: INTRODUCTION

I. Origin of Spontaneous Mutations

A. Replication fidelity and mutagenic DNA repair

The basis for evolutionary change in all organisms is the mutations in their genome. A mutation is a heritable alteration of the nucleotide sequence in the genome, usually DNA. These alterations range from single base substitutions to large chromosomal rearrangements. The infrequent alterations that occur in the absence of treatment with DNA-interactive agents are referred to as spontaneous mutations. Although laboratory conditions may preclude exogenous mutagens, such conditions may not be a reflection of the organism's normal environment. For example, the normal environment for *Escherichia coli* is the mammalian colon, where the bacterial cells may be exposed to exogenous mutagens that are not generated under laboratory conditions (e.g., for cells cultured in LB broth). Only the former situation, however, provides a measure of spontaneous mutation under conditions to which the organism has adapted over time.

It is instructive to examine spontaneous mutations to gain some insight to the fundamental mechanisms through which evolutionary change is driven. We now have the means to do this in different species by determining mutation spectra which provide a profile of the relative frequency of different types of mutations and their distribution within a chosen region of the genome.

The isolation of genetic variants of DNA polymerase in bacteriophage T4, some of which increased (Speyer, 1965) and others decreased (Drake and Allen, 1969) the spontaneous mutation rate, provided evidence for a theory on the origin of spontaneous mutations (Drake, 1969; Drake et al., 1969). Biochemical studies subsequently showed that a 3'→5' exonuclease function in DNA polymerase I could serve to proofread misincorporation errors during polymerization (Brutlag and Kornberg, 1972). From this, it was proposed that mutator and antimutator phenotypes could be ascribed to a ratio of exonuclease to polymerase activity. Mutator alleles were also known in bacteria

(Treffers et al., 1954; Miyake, 1960) and additional loci were soon discovered (reviewed in Cox, 1976), most of which appeared to produce specific base substitutions or frameshift mutations associated with DNA replication.

Concurrently, other investigators isolated radiation-sensitive mutants which also exhibited elevated spontaneous mutation rates (Böhme, 1967; Jyssum, 1968; von Borstel et al., 1968). Also, it had been shown earlier that spontaneous mutation rates for putative frameshift alleles were 6- to 20-fold higher during meiosis than in mitosis (Magni and von Borstel, 1962; Magni, 1963), unlike base substitutions (von Borstel et al., 1964; Magni, 1964; Magni, 1965). These studies suggested that meiotic recombination could be facilitating unequal crossing over at sites of iteration in the DNA, as was predicted (Sax, 1931) long before the structure of DNA was revealed (Watson and Crick, 1953). From these and other findings, it was hypothesized that spontaneous mutations might well derive from DNA repair and recombination, in addition to replication (von Borstel, 1969). This theory was augmented by another line of evidence which directly implicated DNA repair in the production of mutations, namely that mutants in both prokaryotes and eukaryotes existed which, although more sensitive to killing by mutagenic agents, produced fewer mutations among the surviving cells (Witkin, 1967; 1976; Bridges et al., 1968; Lemontt, 1971; Mount et al., 1972; Lawrence et al., 1974).

If spontaneous mutations could arise from lesions or replication errors incurred during DNA metabolism, it remained to be determined by what mechanism this occurred and the relative contribution of the aforementioned processes to a mutation spectrum. Since both DNA repair and recombination entail replication within the process, all mutations may theoretically derive from a deviation of the normal incorporation of nucleotides by polymerases. Such deviation may result in the formation of mismatches between base pairs or the incorporation of nucleotides across from a DNA lesion ("translesion synthesis"). Replication fidelity is thought to be contolled at three levels of enzyme function (Kornberg and Baker, 1991): (1) selection of nucleotides before formation of a phosphodiester bond between a suitable nucleotide and the 3'-terminus of a building duplex DNA chain; (2) proofreading or editing after incorporation; and (3) mismatch repair following elongation of the chain. At the level of base selection, studies with purified DNA polymerases from both eukaryotes and prokaryotes have estimated fidelity to be in the range of 10^{-5} to 10^{-3} errors per replicated nucleotide (Loeb and Kunkel, 1982). This fidelity is 100- to 1000-fold greater than that which would be predicted from the difference in free energy (ΔG) associated with hydrogen bonding between base pairs. By comparison, *in vivo* replication error rates for *E. coli* are about 10^{-10} to 10^{-9} (Drake, 1969). Thus, the remaining fidelity presumably resides with the proofreading and mismatch repair proteins.

Numerous genes have been identified in *E. coli* that encode fidelity factors separate from the polymerase itself (Nevers and Spatz, 1975; Cox, 1976; Scheuermann et al., 1983; Radman and Wagner, 1986; Echols and Goodman, 1991), unlike the situation for DNA polymerase I in which the exonuclease and polymerase activities represent separate domains of a single gene product. Only a few of these genes code for exonucleases. However, the mutator and antimutator phenotypes associated with exonucleases, active only during DNA replication, underlie the biological importance of these enzymes as proofreading components in a multienzyme complex, or replisome (Echols et al., 1983; DiFrancesco et al., 1984; Scheuermann and Echols, 1984). Although purifications of at least two of five types of eukaryotic polymerases retain an associated 3'→5' exonuclease activity (Byrnes et al., 1976; Crute et al., 1986; Syväoja and Linn, 1989), there is no direct proof for an editing function of these enzymes in eukaryotic replication.

The majority of fidelity factors act postreplicatively on DNA containing a mismatch between the two strands. DNA which harbours base pair mismatches or unpaired nucleotides, through either deletion or insertion, is recognised and corrected by various enzymes broadly grouped as mismatch repair. These enzymes, found in both prokaryotes and eukaryotes, remove mismatches associated with homologous recombination and replication of the genome. In *E. coli*, mismatch repair systems are of two types (reviewed in Modrich, 1991). One pathway, controlled by methylation of adenine in GATC sequences in one strand of the DNA, produces large excision tracts of ~1 kilobase in length. Modrich (1991) defines short-patch excision repair, the second system, as DNA repair which leads to excision of segments of less than 10 base pairs in length. This embraces a heterogeneous group of enzymes, including glycosylases and AP endonucleases, most of which remove only one nucleotide.

The relative contribution of the exonucleolytic editing function versus mismatch repair to overall replication fidelity was determined in a study of *dnaQ* (*mutD*) mutants of *E. coli* tested under growing conditions. Spontaneous mutation rates in these mutants are elevated by 10^4- to 10^5-fold (Schaaper and Radman, 1989). Although *mutD5* encodes a defective 3'→5' exonuclease of DNA polymerase III holoenzyme, transformation of this strain with a multicopy plasmid bearing either of two rate-limiting components (*mutH*$^+$ or *mutL*$^+$) of methyl-directed mismatch repair reduced the mutator effect to ~100-fold. Hence, the large increase in mutation rate in an exonuclease-deficient strain is partly due to saturation of mismatch repair, in substantiation of an earlier prediction that the proofreading component exerts no more than a 200-fold increase upon replication fidelity (Fersht et al., 1982). These results indicate that mutation rates in the range of 10^{-10} can be accountable by the fidelity of nucleotide incorporation (e.g., 10^{-5}), exonucleolytic proofreading (e.g., 10^{-2}) and mismatch repair (e.g., 10^{-3}).

The above findings with respect to the fidelity of DNA replication *per se* provides a framework within which to consider the hierarchy of enzymatic steps from which spontaneous mutations could arise. Indeed, the very fact that so many different genes have a direct bearing on spontaneous mutation rates provoked consideration that most spontaneous mutations could be associated with DNA repair, including that of mismatch repair (Hastings et al., 1976). With the discovery of antimutator alleles in a variety of species, this hypothesis focussed on the idea that certain DNA repair processes might create mutations. Hence, the origin of spontaneous mutations was extended to include mutagenic repair of spontaneous lesions (Hastings et al., 1976) which envisioned that formation of intracellular mutagens through normal metabolic processes could generate DNA lesions as one possibility.

The question now arises as to how the inherent spontaneous mutational mechanisms respond to mild and extreme changes in the cellular environment. Such alterations as temperature, exogenous and endogenous oxidative reactions, and salinity cause cellular stresses which can effect the spontaneous mutation rate. For example, it is known that the spontaneous mutation rate in yeast doubles for every five degree increase in ambient temperature between 10°C and 25°C (R. C. von Borstel and C. M. Steinberg, unpublished data). Cellular responses to oxidative and osmotic stresses are outlined below.

B. Apurinic sites and oxidative DNA damage

Amid a flurry of interest in the carcinogenic effects of exogenous mutagens in the environment (Ames, 1971; McCann et al., 1975; Burch, 1976; Cairns, 1976), the presence of intrinsic mutagenic factors within the cell was somewhat overlooked. However, a different perspective began to emerge from studies of the fundamental physical chemistry of the DNA molecule and the demonstration that depurination of DNA was mutagenic (Shearman and Loeb, 1977; 1979).

Depurination and to a much less extent depyrimidination are the most frequently occurring structural changes in DNA under normal physiological conditions, amounting to $\sim 10^4$ events per cell per day (Lindahl and Nyberg, 1972). Although hydrolytic depyrimidination is 100 to 500-fold less frequent than depurination for DNA in neutral solution (Lindahl and Karlstrom, 1973), the relative abundance of abasic sites which derive from either purines or pyrimidines inside the cell may be somewhat different. Other factors are known to contribute to the production of AP sites such as oxidative damage by endogenous free radicals (Hutchinson, 1985; von Sonntag, 1987) and the action of various DNA glycosylases that cleave altered and mispaired bases (Friedberg, 1985; 1991; Sancar and Sancar, 1988). For example, deamination of cytosine to uracil occurs frequently enough inside the cell so that a separate enzyme appears to have evolved to remove this specific mispair. The enzyme, uracil-DNA glycosylase, excises uracil exclusively and leaves behind an AP site (Lindahl, 1979; Crosby et al., 1981).

Given the multitude of mechanisms for the formation of AP sites in DNA under normal physiological conditions and their apparent potential to give rise to mutations, it is prudent to consider that these lesions may underlie the production of a significant fraction of spontaneous mutations. In this context it is interesting to consider that the production of AP sites from a diversity of lesions simplifies the task for their removal through a common enzymatic mechanism and may also afford the cell a global signal for monitoring the general extent of DNA damage.

A variety of AP endonucleases have been identified in many species of prokaryotes and eukaryotes and these are classified as class I or II depending on whether the enzyme cleaves the phosphodiester bond on the 3' or 5' side of the abasic deoxyribose in DNA (Lindahl, 1979; Friedberg, 1985; Sancar and Sancar, 1988). At least two AP endonucleases have been purified from human cell lines (Kane and Linn, 1981; Shaper et al., 1982), and the cDNA encoding one of these enzymes has been cloned and its expression studied in AP endonuclease-deficient bacteria (Demple et al., 1991). The structural gene which codes for the major AP endonuclease in *Saccharomyces cerevisiae* has also been cloned and sequenced (Popoff et al., 1990). Its biochemical properties are not unlike endonuclease IV in *E. coli* which functions both as a class II AP endonuclease and a 3'-diesterase to remove 3'-terminal fragments of deoxyribose which otherwise block repair polymerases at AP sites (Johnson and Demple, 1988a; 1988b; Popoff et al., 1990; Ramotar et al., 1991a).

The endogenous formation of active oxygen species through aerobic respiration is now considered to be among the major contributing factors to the production of spontaneous DNA damage (Hutchinson, 1985; Imlay and Linn, 1988). This idea gained more serious attention following experiments which specifically addressed the mutagenic and chromosome-damaging effects of exogenous sources of oxygen free radicals, such as gamma radiation, hyperbaric oxygen, hydrogen peroxide (H_2O_2), and superoxide-generating drugs (Brawn and Fridovich, 1981; Levin et al., 1982; Moody and Hassan, 1982; Larramendy et al., 1987). Consecutive univalent reduction of molecular oxygen inside the cell generates active oxygen species, such as superoxide ($O_2{\cdot}^-$), H_2O_2, and hydroxyl radicals ($OH\cdot$), presumably as transient intermediates within the enzymes that perform electron transport to O_2. These enzymes include not only the mitochondrial cytochromes involved in oxidative phosphorylation, but also microsomal monooxygenases and dioxygenases which function to oxygenate various organic substrates and to desaturate fatty acids. Oxidative damage to DNA can give rise to AP sites; however, all components of the primary structure of DNA are susceptible to oxidative attack by free radicals generating as many as 100 different fragmented products (Hutchinson, 1985; von Sonntag, 1987; Imlay and Linn, 1988).

II. Osmotic Stress

The availability of water is of critical importance to the survival of living cells. From a biochemical perspective, the principal components in the make-up of all organisms (i.e., proteins, nucleic acids and lipids) derive their properties and function only within this medium. High solute concentrations outside the cell decrease the intracellular availability of water through osmosis, producing an osmotic (or water) stress.

The osmotic property of a given solution can be described in terms of the osmotic potential (p), where p is defined by the equation:

$$p = (RT/V) \ln a$$

and R = the universal gas constant, T = absolute temperature, V = the partial molar volume of water and a = water activity (or ratio of the number of water molecules to the total number of molecules for both solute and solvent). Since the addition of solutes to a solvent reduces the solvent activity to values below 1.0, distilled water should have an osmotic potential of 0 and aqueous solutions have osmotic potentials with negative values. Osmotic pressure (π) is also frequently used to describe the osmotic property of solutions, as defined by the equation:

$$\pi = M\,R\,T$$

where M = molarity. It is apparent from these equations that a solution with low osmotic pressure is one with high osmotic potential. Osmosis is a phenomenon which causes water to move across a semipermeable membrane from a compartment with high osmotic potential (or water activity) to one with a lower value.

The primary agents of osmotic stress are dessication, freezing and fluctuating salinity. Over time, selective pressure has been imposed on organisms subject to osmotic stress so that all organisms to this day have evolved various strategies to cope with variation in external osmolarity. Mechanisms for osmotic adaptation in a wide range of species have come to light in recent years (Yancey et al., 1982; Le Rudulier et al., 1984), stemming from early inquiry into the fundamental process of osmosis in living systems (Potts, 1968; Scholander, 1975) and studies of the wide-ranging capacity of different species to grow in environments with low water activity (Kushner, 1978; Larsen, 1986).

Organisms have been grouped according to their ability to proliferate in environments with different water activity. Halotolerant and halophilic species are those which show tolerance versus preference for high salt concentrations (generally, but not always, NaCl). Xerotolerant species are those which are well-adapted to conditions of dessication, such as found in a desert. On the basis of halotolerance in microorganisms, Larsen (1986) recognizes 4 broad groups, ranging from nontolerant (which cannot proliferate much beyond 1% or ~0.17 M NaCl) and slightly tolerant (inhibited above 6-8% or ~1.0-1.4 M NaCl) to extremely tolerant species (withstand salt concentrations from zero to saturation). Microorganisms which have evolved the ability to grow in either extreme of water activity, such as a mountain spring or a saturated brine (Hocking, 1988), tend to be more confined to these habitats, presumably because the optimum range of function of intracellular enzymes has shifted to a different mean value for the intracellular water activity. In this respect, most of the common potential contaminants of foods are nontolerant of salt, and this is the basis for using salt as a means of preserving foods.

The adaptive response to a reduced water activity is remarkably similar among different species. Osmotically stressed cells accumulate a restricted group of low molecular weight solutes. These compounds have been termed compatible solutes (Brown and Simpson, 1972) because they can be accumulated to a substantial intracellular concentration (e.g., up to 1 molar in some cases) without severely affecting cellular processes. When cells are subjected to an increase in external osmolarity, the immediate consequences are a loss of turgor pressure (if a cell wall is present) and a reduction in cytoplasmic volume (i.e., plasmolysis) through osmosis. The cell responds to this stress stimulus by increasing the production of intracellular solutes, which lowers the internal water activity and reverses the outflow of water. Thus, the cell volume and turgor pressure are restored to their previous levels.

Compatible solutes (also termed osmolytes or osmoprotectants) are ideally small uncharged molecules that are neither obligatory to metabolism nor permeable to the plasma membrane so that they can be effectively accumulated within the cell. Among various phyla of organisms, these substances include some sugars (sucrose, fructose), sugar alcohols (glycerol, erythritol, arabitol, sorbitol, mannitol), glucosylglycerols (floridoside, isofloridoside), certain amino acids (proline, glycine, alanine, glutamic acid, glutamine, γ-aminobutyrate), and their N-methylated derivatives (glycine betaine, choline, proline betaine, trimethyl-γ-aminobutyrate) (Flowers et al., 1977; Yancey et al., 1982; Le Rudulier et al., 1984; Imhoff, 1986; Csonka, 1989). Glycerol is often freely diffusible across the plasma membrane which limits its effectiveness as an osmolyte, although some species have developed mechanisms of increasing the retention of glycerol under conditions of osmotic stress (Adler et al., 1985; Hosono, 1992). Potassium ions are among the few inorganic ions that are known to act as osmolytes in many species of bacteria (Christian and Waltho, 1961; Reed and Stewart, 1985; Epstein, 1986). Their effectiveness is limited to counteracting low levels of osmotic stress because high concentrations of K^+ become inhibitory to enzyme function (Bowlus and Somero, 1979). As the severity of osmotic stress increases, bacteria turn to production of the uncharged osmolytes, notably proline (Measures, 1975), glycine betaine (Le Rudulier and Bouillard, 1983; Perroud and Le Rudulier, 1985; Le Rudulier and Bernard, 1986), choline (Landfald and Strøm, 1986) and analogs of these compounds. These same osmolytes are utilized by various species of vascular plants (Storey and Wyn Jones, 1975; Hanson and Hitz, 1982; Morgan, 1984) and marine invertebrates (Clark, 1968; Burton, 1991). A wide range of taxonomic groups including fungi (Brown, 1976), algae (Wegmann, 1986), insects (Salt, 1957), crustaceans (Conte et al., 1977), some vascular plants (Cutter et al., 1977) and vertebrates (Schmid, 1982) utilize sugars or polyhydric alcohols as the principal osmolyte. The emerging picture from the cumulative studies of osmolyte systems in different organisms is that only a small set of molecules appears to have suitable properties to act as an osmolyte. This comparison suggests a remarkable degree of convergent evolution (Yancey et al., 1982).

When organisms are exposed to conditions of hyperosmotic shock, the immediate period of plasmolysis is inhibitory to physiological processes such as DNA replication (Meury, 1988) and active transport (Roth et al., 1985a; 1985b; Walter et al., 1987). Ohwada and Sagisaka (1988) have observed a sharp increase in ATP levels in response to osmotic upshock, which Csonka (1989) suggests might represent the net effect of an overall inhibition of biosynthesis. In this respect, various studies of enzyme function *in vitro* have shown that enzyme activities decline as the concentration of NaCl or KCl rises beyond 0.10 to 0.15 M (Borowitzka and Brown, 1974; Stewart and Lee, 1974; Bowlus and Somero, 1979; Zimmerman and Harrison, 1987). The inorganic ions alter enzyme function by lowering V_{max} or raising K_m. Zimmerman and Harrison (1987) have taken these ideas somewhat closer to the *in vivo* situation by examining the effects of macromolecular crowding together with increased salt concentration. Their studies with DNA polymerase I show that macromolecular crowding increases the binding affinity of this enzyme with its template primer so that the optimum activity is shifted toward a higher salt concentration. In the absence of macromolecular crowding, K^+ ions inhibit the activity of DNA polymerase I in much the same manner as the *in vitro* studies have shown for enymes of intermediary metabolism. These findings suggest that enzyme function *in vivo* under conditions of reduced cell volume may not be as adversely affected by inorganic ions as was previously predicted.

The protective effect of osmolytes is thought to occur by stabilization of the native conformation of proteins (Gekko and Timasheff, 1981; Arakawa and Timasheff, 1985; Low, 1985). By increasing the surface free energy of water, osmolytes are preferentially excluded from the hydrated surface of proteins. Thermodynamically, the exclusion of solutes from the water-protein interface favours intramolecular interactions within the tertiary protein structure or subunit assembly between proteins rather than interaction with the solvent (i.e., unfolding of tertiary structure).

A. Osmotic adjustment in yeast cells

Most species of fungi adapt to conditions of increased osmotic pressure by increasing the intracellular concentration of polyhydric alcohols (Brown, 1978; Adler and Gustaffson, 1980; Luard, 1982; Gadd et al., 1984; Hocking, 1986). The yeasts generally accumulate glycerol, although the mechanisms for osmotic adjustment with glycerol are somewhat different between halotolerant and nontolerant species. *Saccharomyces cerevisiae*, which is relatively intolerant of high salt concentrations, increases the rate of synthesis of glycerol but loses a constant proportion by external diffusion (Edgley and Brown, 1983; Reed et al., 1987). On the other hand, halotolerant species, such as *Zygosaccharomyces rouxii* and *Debaryomyces hansenii*, actively take up glycerol (Adler et al., 1985; van Zyl et al., 1990) and alter the lipid chemistry of the plasma membrane (Hosono, 1992) in response to hypertonic conditions. In the presence of NaCl, the cellular membranes of *Z. rouxii* show fewer unsaturated fatty acids, thus decreasing membrane fluidity, and a greater protein content. Both of these factors could account for the increased retention of glycerol.

When cells of *Saccharomyces cerevisiae* are exposed to an osmotic upshock by transfer to a medium supplemented with 2.28% (0.39 M) NaCl the cell volume is decreased within the first hour after transfer, but between 1 to 3 hours the volume is almost restored to pre-stress levels (Meikle et al., 1988). Increasing the external concentration of NaCl increases the lag time before production of osmotically induced glycerol (André et al., 1988). For example, glycerol accumulation is delayed until 6 hours after transfer to 8% (1.37 M) NaCl (Brown et al., 1986). Upon restoration of turgor pressure, cell growth can ensue. When NaCl is present in the medium, glycerol is synthesized throughout the logarithmic phase of growth but declines as the population approaches stationary phase (Meikle et al., 1988). Instead, the onset of stationary phase is accompanied by the synthesis of trehalose, which appears to replace the abundance of glycerol. Production of this disaccharide occurs only when the supply of glucose is depleted (Panek, 1985) and is not osmotically mediated (i.e, trehalose is produced at stationary phase with or without growth in hyperosmotic medium). Although intracellular production of trehalose is not necessarily mediated by stress stimuli, it is often produced in spores or resting cells that are apt to experience stress conditions (Thevelein, 1984; Van Laere, 1989). In *S. cerevisiae*, several studies have shown that trehalose content is correlated with resistance to dehydration (Gadd et al., 1987), hyperosmotic shock (Mackenzie et al., 1988), heat shock (Hottiger et al., 1987) and agents that induce the heat shock response (Atfield, 1987). Unlike the response to hyperosmotic stress, heat shock triggers an immediate and rapid production of trehalose.

B. Comparison of osmotic adjustment in *E. coli* and *S. cerevisiae*

Osmotic adjustment in *Escherichia coli*, unlike *Saccharomyces cerevisiae*, involves accumulation of potassium and glutamate ions, and secondarily the organic solutes, chiefly proline and glycine betaine. The effect of hyperosmotic shock on K^+ and H^+ ion flux across the membrane is quite different between these two microorganisms and may form the underlying basis for the accumulation of amino acids versus polyols as osmoprotectants. The accumulation of potassium ions in *E. coli* (from intracellular concentrations of 0.15 M up to 0.55 M) is dependent upon the osmolarity of the growth medium (Christian and Waltho, 1961; Epstein and Schultz, 1965). Hyperosmotic shock activates uptake of K^+ ions by two constitutive transport systems, Kup and TrkA (Epstein, 1986; Bakker et al., 1987; Bossemeyer et al., 1989), and induces synthesis of a higher affinity system encoded by the *Kdp* operon (Laimins et al., 1981). Dinnbier et al. (1988) have shown that accumulation of K^+ and the associated efflux of H^+ (which increases cytoplasmic pH) are only transient and that after 30 minutes the flux is reversed. These effects occur independently of the presence of a carbon source. The situation in *S. cerevisiae* appears to be reversed, where the addition of glucose to osmotically stressed cells leads to a short term decrease in the internal potassium concentration which is later reversed (Brown, 1990). In the absence of osmotic stress, glucose uptake is normally associated with H^+ efflux and K^+ influx.

A few genes have been isolated in *S. cerevisiae* which, among other pleiotropic effects, are associated with osmotic sensitivity. The principal plasma membrane H^+-ATPase gene (*PMA1*) has been cloned and exhibits homology with various H^+/K^+, Na^+/K^+ and Ca^{2+}-ATPases including the bacterial kdpABC K^+-ATPase (Serrano et al., 1986). Mutations in this gene affect pH, cold and osmotic sensitivity (McCusker et al., 1987). Gaxiola et al. (1992) have isolated genes which specifically confer halotolerance to NaCl in yeast. Overexpression of *HAL1* yields improved growth under salt stress conditions and disruption of the gene decreases salt tolerance. Although antibodies specific to this gene product localize to the cell membrane, *HAL1* does not code for a transport protein. Homologous counterparts of this gene are present in plants (R. Gaxiola and R. Serrano, in preparation) and both plants and fungi regulate the intracellular levels of K^+ in response to different external factors by modulating the uptake of K^+. Thus, *HAL1* might be a regulatory factor for K^+ transport.

The accumulation of K^+ ions in *E. coli* induces synthesis of glutamate (Measures, 1975) which may act as a counterion, in conjunction with the expulsion of H^+ ions, to maintain the membrane potential within the range of -120 to -150 mV (Castle et al., 1986; Csonka, 1989). Although the increased supply of glutamate could be used to synthesize proline, a number of studies have shown that gram-negative bacteria cannot accumulate proline or glycine betaine under conditions of osmotic stress unless it is available in the medium (Le Rudulier and Bouillard, 1983; Csonka, 1988). Thus, biosynthesis of proline does not appear to be the mechanism of osmotic adjustment. Subsequent work has identified three proline permease systems, PutP, ProP and ProU (Wood, 1988), one of which (PutP) is utilized for uptake of proline as a carbon or nitrogen source (Ratzkin et al., 1978) and the other two (ProP and ProU) are activated under conditions of hyperosmotic stress for uptake of proline as an osmolyte (Cairney et al, 1985a; Dunlap and Csonka, 1985; Grothe et al., 1986; Milner et al., 1988). Interestingly, the transport of glycine betaine has proven to be mediated by the ProP and ProU systems as well (Cairney et al, 1985b; May et al., 1986), even though its structure is quite unlike that of proline.

In addition to the genes which encode the transport proteins listed above, there are many other genes in *E. coli* that are subject to regulation of expression by osmotic conditions. These include approximately 20 genes, notably the porin genes, *OmpF* and *OmpC* (which encode two kinds of membrane channels that facilitate diffusion of hydrophilic molecules) and their regulatory genes, *OmpR* and *EnvZ*, the *bet* genes (encoding enzymes for oxidation of choline to glycine betaine), *ots* (trehalose biosynthesis), and *tre* (transport and metabolism of trehalose). Studies describing the cloning, sequencing and mechanism of expression of these genes in fusion constructs with *lacZ* are reviewed in Csonka (1989).

The mechanism for osmotic adjustment in *E. coli* and other enteric bacteria as described above is clearly different from that in *S. cerevisiae*, which redirects part of the metabolism of glucose and other sugars gained from the medium to the synthesis of copious amounts of glycerol. Evidence in support of altered synthesis is indicated by the increase in specific activities of glycerol-3-phosphate dehydrogenase and phosphofructokinase upon exposure to osmotic stress (Edgley and Brown, 1983). No studies have addressed the possibility that glycolytic enzymes which affect the production of glycerol are also subject to regulation of transcription or translation through osmotically induced signals.

C. Osmoregulatory signals

Although the physiological mechanisms for osmotic adjustment to variation in water activity have been described for a wide range of species, and with considerable insight at the molecular level for species such as *E. coli*, it is still not known what constitutes the fundamental signals of osmotic variation that elicit these adjustments. Possibly, changes in turgor pressure may be detected by a membrane-bound protein which functions as a pressure sensor (Csonka and Hanson, 1991). Such a mechanism might work for those species (bacteria, algae, fungi, plants) in which a cell wall can provide the necessary rigidity to maintain intracellular hydrostatic pressure. However, it is not entirely clear that turgor pressure necessarily drives cell wall expansion in organisms with cell walls (Milner et al., 1988) and osmoregulation is clearly evident in cells without walls, such as erythrocytes (Kregenow, 1981). Another possibility is that a cytoplasmic protein may respond to changes in concentration of a specific intracellular component.

III. The Stress Response and Mutagenesis

A. Stress proteins

The adaptive response of yeast cells, other eukaryotes and prokaryotes to conditions of low water activity has apparently evolved among those species which frequently encounter this type of stress stimulus in their environment. Such conditions could be fundamental to the survival of most species, especially terrestrial ones, and therefore mechanisms which prevent the damaging effects of anhydrobiosis are germane to all living cells. These mechanisms, presented in the previous section (and reviewed by Crowe et al., 1992), have tended to focus on damage to proteins and lipids. Few studies have considered whether DNA and RNA might also sustain damage under conditions of extreme water stress and in relation to protective measures which counteract the denaturation of biological macromolecules. However, the mutagenic effects of other physiological stress conditions, such as heat (Kricker and Drake, 1990), nucleotide pool imbalance (Barclay et al., 1982), starvation (Ilyina et al., 1986), and aerobic utilization of oxygen (Moriya et al., 1991; Shibutani et al., 1991; Reid and Loeb, 1992) have been described.

Stress produces physiological conditions which cause damage to cellular macromolecules either directly or indirectly. These conditions have been seen to elicit the synthesis of a group of proteins, called stress proteins, which may serve to protect the cell from further damage. The production of heat shock proteins was first described by Ritossa (1962) who observed that a mild heat shock caused the appearance of specific puffs in the polytene chromosomes of *Drosophila busckii.* Increased transcription of specific genes in response to heat has proven to be characteristic of all organisms (Lindquist and Craig, 1988; Watson, 1990) and it has become apparent that environmental stimuli other than heat can induce transcription of the same proteins. Hence, the heat shock proteins have come to be referred to more generally as stress proteins. Although some of these proteins are common to different stress conditions, the profile of proteins induced is by no means homogeneous. As well, the transcription of several gene products is terminated (Kapoor, 1986).

Conditions, apart from heat shock, which induce stress proteins include starvation (for either carbon or nitrogen sources), uv irradiation, and exposure to dinitrophenol, nalidixic acid, arsenite, $CdCl_2$, alcohols (ethanol, methanol, butanol), antibiotics (puromycin, paromomycin, tetracycline), amino acid analogs (canavanine), and reactive derivatives of oxygen (Watson, 1990). This list includes only a few of the more prominent stress conditions reported from a wide range of organisms. Some of these agents are well known to cause damage to DNA. In yeast, studies have shown that heat shock and the stress response so induced can increase the resistance to ultraviolet and ionizing radiation (McAlister and Finkelstein, 1980). Subsequent work indicated that this resistance to radiation could be explained by the induction of recombinational repair though not excision repair (Mitchel and Morrison, 1982a,b; 1983).

Stress proteins have been categorized into three groups according to size. These groups often share considerable similarity in structure between divergent species (Lindquist and Craig, 1988; Rose et al., 1989; Normington et al., 1989) which suggests that they perform fundamental cellular processes. One of the most highly conserved of all eukaryotic proteins and smallest of the stress proteins (8.5 kDa) is ubiquitin (Sharp and Li, 1987; Özkaynak et al., 1987). This protein, like many of the stress proteins, is present under normal physiological conditions in which it has been associated with a range of cellular processes including DNA repair, cell cycle control, cellular differentiation and formation of ribosomes and peroxisomes (see Finley and Chau, 1991 for review of other putative functions). In each of these processes and in the stress response, ubiquitin appears to function in the proteolytic turnover of proteins. Covalent attachment of ubiquitin to acceptor proteins, catalyzed enzymatically (Ciechanover et al., 1982; Pickart and Rose, 1985), instigates their degradation by an ATP-dependent multienzyme complex (Herschko et al., 1980; 1984). The isolation and examination of a conditional-lethal murine cell line, ts85, carrying a mutation in a ubiquitin-activating enzyme, showed that ubiquitin is essential for progression through the cell cycle and for cell survival (Finley et al., 1984; Ciechanover et al., 1984). Accumulation of "short-lived proteins" (cf., "long-lived proteins", which are largely removed by lysosomal degradation) in these cells suggested a proteolytic role of ubiquitin in their removal. Many of these proteins may be regulatory factors necessary for proper control of the cell cycle and other processes.

In *S. cerevisiae* a multigene family, *UBI1-UBI3*, maintains production of ubiquitin under normal growing conditions, but in response to stress, a separate gene, *UBI4*, consisting of 5 tandem repeats of the ubiquitin element within a single open reading frame (ORF), increases the supply of ubiquitin (Özkaynak et al., 1987; Finley et al., 1987). Deletion of the *UBI4* gene abolishes the cell's capacity to resist stress conditions (Finley et al., 1987). In prokaryotes there is no homologous counterpart for ubiquitin, but the *lon* gene appears to code for an analogous proteolytic function. *E. coli* strains that lack the stress-inducible *lon* gene fail to degrade abnormal proteins (Kowitt and Goldberg, 1977).

Stress proteins belonging to the hsp58, hsp70 and hsp83 groups are highly conserved in both prokaryotes and eukaryotes (Lindquist, 1986; Watson, 1990). The induction of these proteins in response to a variety of stress treatments is attributed to the accumulation of denatured (unfolded) proteins. This was concluded in one study which tested the transcriptional activity of the *hsp70* gene in a fusion gene between *E. coli lacZ* and the promoter region of *Drosophila hsp70* (Ananthan et al. 1986). Micro-injection of denatured proteins together with the fusion gene into frog oocyte nuclei resulted in a 10-fold increase in the expression of β-galactosidase, but there was no effect on transcription in response to undenatured proteins.

Recent evidence suggests that the above stress proteins function during normal cell growth in the assembly of oligomeric proteins and in the translocation and subsequent refolding of proteins imported to cellular compartments (Pelham, 1986). For example, mutants of *hsp60* in *S. cerevisiae* accumulate incompletely processed proteins within the mitochondrial matrix (Cheng et al., 1989; Ostermann et al., 1989), from which it would seem that *HSP60* normally functions in the refolding of mitochondrial proteins following translocation across the membrane. Thus, as in the case of ubiquitin, the range of functions of the larger stress proteins, originally detected as inducible factors with a protective role in response to heat, extends to normal physiological processes in the cell as well.

B. Investigation of mutagenesis and recombination due to osmotic stress

The initial impetus for this study came from findings which indicated that NaCl, $MgCl_2$, and various salts of saccharin appeared to be clastogenic in mammalian cells for reasons that could not be reasonably explained by the reactivity of these compounds with DNA (Ashby, 1985; Ashby and Ishidate, 1986). Also, it was known that NaCl could induce mutations and recombination in yeast when the cells were exposed to high concentrations in the range of 2 to 4 molar (S. Sora and G. E. Magni, unpublished data). As a test of the hypothesis that solutes, ordinarily unreactive to DNA, were mutagenic as a function of osmolality, several monovalent and divalent salts plus a few non-ionic solutes were tested at equivalent osmolalities for mutagenicity in a haploid strain of yeast. This led to the conclusion that the mutagenic effects were a manifestation of osmotic plasmolysis. It was also apparent that only logarithmic phase cells were sensitive to mutation induction and lethality caused by osmotic shock. These findings are presented in part I-A of chapter 3, and are also published (Parker and von Borstel, 1987).

Chapter 3 (I-A) also describes experiments in which lower salt concentrations were tested to see if a threshold level of osmolality was required for the expression of this type of mutagenesis. Although a threshold was identifiable in which the lower limit of the osmotic phenomenon was marked by antimutagenesis, these experiments revealed further complexity of the mutagenic response. For example, reducing the osmotic shock from 2.0 to 1.0 M NaCl appeared to greatly delay the response. Secondly, some solutes (saccharin, NH_4Cl) were identified that caused greater expression of mutations by osmotic shock than would be predicted on the basis of osmolality. Further dose-action experiments (completing I-A) were conducted to substantiate the observation that both the lethal and mutagenic effects of a severe osmotic shock could be reduced by keeping the cells in the growth medium (cf., replacing the medium with a hypertonic solution). This appeared to have direct bearing on physiological work on the mechanisms by which all organisms, including yeast, cope with a stress situation involving low water activity.

Experiments describing the effects of osmotic shock upon recombination in diploid yeast are presented in section I-B of chapter 3. Although a very large increase in mitotic recombination was observed, it was quite surprising to find that the survival of diploid strain D7-144 was considerably lower than that of haploids for the same level of osmotic shock treatment. Subsequent experiments determined that other diploid strains (including strain D7, which unlike D7-144, is sporulation-proficient) exhibit a similar sensitivity to osmotic shock as haploid strains.

A different approach was taken in part II to investigate the more subtle effects osmotic stress might have upon mutation rates at lower concentrations and under growing conditions. Using a method of measuring mutation rates which captures the revertants as they arise during the growth of the cell population, it was determined that concentrations as low as 0.30 M NaCl do increase the spontaneous reversion rates of certain alleles in haploid yeast. These measurements were substantiated by two other methods for measuring mutation rates, as summarized in part II-A. To ascertain the basis for this increase in mutation yield, a system was utilized whereby forward mutations occurring at the *URA3* gene in yeast could be selected and then sequenced. The spontaneous mutation spectra for *ura3*$^{-}$ mutations obtained under normal and hypertonic conditions are presented in part II-B. When it was observed that the distribution of specific classes of base substitution was somewhat biased across the open reading frame of the *URA3* gene, experiments were devised (in II-C) that could possibly explain the mutational specificity as a property of DNA repair.

The basis for the detection of different mutations by the reversion and forward mutations sytems used in this study are described in the next section.

IV. Assays Used to Detect Mutations and Recombination in Yeast

A. Reversion systems

Strain XV185-14C was frequently used throughout this study to detect point mutations, including both frameshift mutations and base substitutions. Its genotype is *ade2-1*, *arg4-17*, *his1-7*, *hom3-10*, *lys1-1* and *trp5-48*. These genetic markers have been crossed into various other strains which were also used in this study (see Table 1). The mutational nature of these alleles is as follows: *ade2-1*, *arg4-17*, *lys1-1* and *trp5-48* are ochre-suppressible chain-terminating alleles, *his1-7* bears a missense mutation that undergoes primarily intragenic suppression, and *hom3-10* harbours a frameshift mutation that can be suppressed externally or by reversion at the locus site.

The chain-terminating alleles harbour a nonsense mutation of the ochre (UAA) type (Gilmore et al., 1971; Gesteland et al., 1976). These alleles can undergo reversion by mutationally altered tRNAs or by reversion at the site of the ochre codon itself. Evidence for external suppression by tRNA molecules can be seen by simultaneous reversion of all of the ochre alleles within the same strain, although not all suppressors act in this manner. An ochre suppressor tRNA (SUP4-o) was sequenced and found to be due to a mutation (G•C→T•A) in the wobble position of the anticodon loop of $tRNA^{Tyr}$ (Goodman et al., 1977). Other suppressors like SUP4-o have been found which map to eight different genes, all coding for $tRNA^{Tyr}$ and all exerting strong suppression of each of the above alleles (Sherman, 1982). Ono et al. (1979a; 1979b) showed that it was possible to select for suppressors at other tRNA genes, such as those which encode serine and leucine, in strains which carry psi factor (ψ^+ strains). It is thought that psi may be an extrachromosomal plasmid, possibly carrying a gene that enhances translational efficiency, but the nature of this element and its gene product remains a mystery (Cox, 1988). Based on this information it is apparent that several different amino acids can substitute for the ochre site to produce a functional enzyme. For *arg4-17*, the ochre site (White et al., 1985) corresponds to a lysine codon in the wild type sequence for *ARG4* (Beacham et al., 1984), which is different again from any of the amino acid replacements associated with suppression.

Suppressor mutations can be distinguished from locus revertants based on the finding that *ade2-1* mutants accumulate a red intermediate, phosphoribosylaminoimidazole, which causes the colonies to be red (Smirnov et al., 1967). Thus, if the medium contains a limiting concentration (typically 5 μg/ml) of adenine, reversions at the locus of *arg4-17* or *lys1-1* will appear red but those due to suppression, which acts on *ade2-1* as well, will be white. In the case of *trp5-48*, a large proportion of the spontaneous revertants are red, unlike *arg4-17* or *lys1-1*. The reason for this difference is not clear, but some of the red TRP$^+$ colonies could represent suppressors that act more specifically at this locus. Characterization of red *ARG4*$^+$ colonies has revealed that a portion of these isolates are suppressors that also suppress *trp5-48* but not *ade2-1*. Hence, the method of differentiating locus revertants from suppressors requires that red colonies be checked by replica plating.

Locus reversion at *arg4-17* could come about, in theory, by one of 7 different base substitutions in the ochre codon (Figure 1), only 2 of which have not been verified by sequence information or knowledge of suppression by serine or leucine tRNAs in ψ^+ strains. This could easily be checked by sequencing a collection of locus revertants. From Figure 1, it can be deduced that only 2 types of transversion (A•T→C•G and A•T→T•A), with equal probability, and 1 transition (A•T→G•C) will insert amino acids at the ochre site. In theory, transversions are more likely to be recovered than transitions by a 6:1 ratio (i.e., assuming random substitutions), but this does not take the sequence context into consideration which may influence the relative frequencies of these mutations. Suppression at *arg4-17* or *lys1-1* in ψ^- strains that gives rise to large white colonies is confined to G•C→T•A or A•T→T•A transversions in one of the 8 tRNA^{Tyr} genes, because all other suppressors exhibit weak suppression at *ade2-1* which causes the colonies to be red. All of the strains used in this study are ψ^-.

The heterogeneity of phenotypes among *HIS1*$^+$ revertants (see Figure 18), osmotic remediability of *his1-7* mutants at 18°C (Hawthorne and Friis, 1964) and identification of second-site suppressors by intragenic mapping (Lax and Fogel, 1978) indicates that reversion of *his1-7* comes about by intragenic missense suppression. The phenotypes are of two types, feeders and nonfeeders, wherein the former is due to an overproduction of histidine. The *HIS1* gene of *Saccharomyces cerevisiae*, which encodes the first enzyme (ATP phosphoribosyltransferase) in the biosynthesis of histidine, has been

FIGURE 1:

Possible base substitutions for reversion of *arg4-17*.
[A] The possibilities for reversion of the ochre codon at position 43 in the ORF of *arg4-17* are indicated together with the corresponding amino acid replacements in the upper panel. The first line depicts the 2 possible base substitutions involving T's, the second and fourth lines show the single possibility for substitution by A and G respectively, and the third line indicates the 3 sites which can be changed to C. Amino acids shown in brackets are unconfirmed as tolerable replacements at this position in the protein; all other amino acids have been confirmed by either DNA sequencing or knowledge of suppression. Codon 43 in wild type yeast is AAA (lys) (Beacham et al., 1984). **[B]** The possibilities for creation of a suppressor mutation in the anticodon of 7 different tRNA genes are shown in the lower panel (e.g., the first line depicts the 2 tyrosine isoacceptors which can undergo A•T → T•A or G•C → T•A transversions to form class I and II suppressors).

A

arg4-17	→	*ARG4*$^{+}$			
TAA		-TT	—	Leu	Tyr
(codon 43)		A--	Lys	—	—
		CCC	(Gln)	Ser	Tyr
		G--	(Glu)	—	—

B

sup^{-}		→	*SUP*$^{+}$	
$\text{tRNA}^{\text{Tyr}}_{\text{UA}\underline{\text{U}}}$	$\text{tRNA}^{\text{Tyr}}_{\text{UA}\underline{\text{C}}}$		$\text{tRNA}^{\text{Tyr}}_{\text{UA}\underline{\text{A}}}$	[I, II]
$\text{tRNA}^{\text{Ser}}_{\text{U}\underline{\text{C}}\text{A}}$			$\text{tRNA}^{\text{Ser}}_{\text{U}\underline{\text{A}}\text{A}}$	[III]
$\text{tRNA}^{\text{Leu}}_{\text{U}\underline{\text{U}}\text{A}}$			$\text{tRNA}^{\text{Leu}}_{\text{U}\underline{\text{A}}\text{A}}$	
$\text{tRNA}^{\text{Lys}}_{\underline{\text{A}}\text{AA}}$			$\text{tRNA}^{\text{Lys}}_{\underline{\text{U}}\text{AA}}$	
$\text{tRNA}^{\text{Glu}}_{\underline{\text{G}}\text{AA}}$	$\text{tRNA}^{\text{Gln}}_{\underline{\text{C}}\text{AA}}$		$\text{tRNA}^{\text{Glu, Gln}}_{\underline{\text{U}}\text{AA}}$	

sequenced (Hinnebusch and Fink, 1983). The *his1-7* allele represents a transition from G to A at position 798 which changes methionine to isoleucine (Savage et al., 1992). In the same study, 38 *HIS1*$^+$ revertants were sequenced. Apart from one revertant at position 798, all of the revertants are due to second site forward mutations within the gene. These data are presented in part II of results (see Table 19), from which it can be seen that among the two classes of *HIS1*$^+$ revertants all of the possible transitions and transversions are represented. Approximately half (17/37 = 46%) of the base substitutions are transitions. Interestingly, different base substitutions for one site within the same codon, which give rise to different amino acid replacements, can generate feeders and nonfeeders.

The wild type *HOM3* gene (Rafalski and Falco, 1988) and the mutant *hom3-10* allele have been cloned and sequenced (Wang et al., 1990; 1991). The mutation in *hom3-10* is a +1 insertion of C in a proline codon, 7 residues upstream from the stop codon. A sequence study of 21 revertants of the *hom3-10* allele determined that the majority (19/21 = 90%) are due to forward mutations in tRNA genes and the remaining fraction (2/21 = 10%) is represented by -1 deletions at or near the site containing the additional base. Frameshift suppression is primarily due to altered tRNAs which permit a 4-base translocation of mRNA on the ribosome (Gaber and Culbertson, 1982; Cummins et al., 1982), such that codon recognition by frameshift suppressor tRNAs does not require pairing at the fourth position (Gaber and Culbertson, 1984). Extragenic suppressors of frameshift alleles bearing +1 insertions in proline codons map to at least 9 different loci (Gaber et al., 1983), 6 of which encode 2 different proline tRNA isoacceptors (Cummins et al., 1985). One such suppressor, *SUF2*, produces suppressor mutations by a base insertion of G within the 3'-GGA-5' anticodon; whereas, isoacceptors encoded by *SUF7*, *SUF8*, *trn1* and 2 unmapped tRNAs, all of which contain introns, arise by a G→T transversion at the 3' splice junction of the intron. This base substitution is thought to disrupt the normal G•C base pairing in the anticodon stem so that the size of the anticodon loop is expanded to read a 4 base codon (Cummins et al., 1985; Winey et al., 1986). Three other suppressors (*suf12*, *suf13* and *suf14*) show cross suppression of different types of frameshift or nonsense mutations (Gaber et al., 1983). Strain XV195-6B was constructed in our laboratory to test for simultaneous suppression of different frameshift alleles. The strain contains three mutant frameshift alleles, *leu2-3* and *his4-38*, both of which contain +1 G•C insertions in glycine codons, and *hom3-10*. Only a minor fraction (3/800) of revertants tested by replica plating are LEU2$^+$, HIS4$^+$, and HOM3$^+$ (U.G.G. Hennig, pers. commun.). The nature of the mutations which produce non-group-specific suppressors is unknown. In summary, the *hom3-10* allele can undergo reversion by G•C→T•A transversions and +1 G•C insertions in suppressor genes, and by -1 base deletions (and possibly +2 base additions) within the locus itself.

B. Forward mutation at the *URA3* gene

There are, at present, two forward mutation systems described for generating mutation spectra in yeast. One detects mutations in a tRNA suppressor gene, *SUP4*-o (Pierce et al., 1987), and the other in a gene required for *de novo* synthesis of uracil, *URA3* (von Borstel and Lee, 1985). Forward mutations in the *SUP4*-o suppressor allele, carried on a centromeric plasmid, abolish suppression at three ochre alleles, *can1-100*, *ade2-1* and *lys2-1*. The mutants are then selected as red colonies on medium containing canavanine and a limited supply of adenine. Mutations in the *URA3* gene have been measured in strains which carry the *URA3* marker on an integrating plasmid and harbour either a disruption (Lee et al., 1988; 1992) or a deletion of the genomic copy. More recently, we have turned to using PCR amplification of the resident copy of the *URA3* gene on chromosome V to examine forward mutations of this gene in any strain of interest. In either case, forward mutations in the *URA3* gene confer resistance to 5-fluoroorotic acid (5-FOA) and are selected on medium containing uracil and 5-FOA.

In *Saccharomyces cerevisiae*, the biosynthesis of pyrimidine nucleotides (UTP, CTP and TTP) occurs by either *de novo* synthesis of uridine-5'-phosphate (UMP) from glutamine and aspartic acid or salvage of exogenous pyrimidines. Fluorinated pyrimidines are toxic to cells through formation of 5-fluoro-UMP; however, resistance can be conferred by mutations in pyrimidine metabolism which block the synthesis of UMP. From orotic acid, synthesis of UMP occurs in two steps catalyzed by enzymes encoded by the *URA5* and *URA3* genes. Mutations in these genes predictably avert the toxicity of 5-FOA, but only a few mutations in the *URA5* gene are recovered in comparison to *URA3* (Boeke et al., 1984; Lee et al., 1988). The reason for the low recovery of *ura5*$^-$ mutants is not clear, but de Montigny et al. (1990) have recently uncovered an isoenzyme of URA5, encoded by *URA10*, which appears to account for the leaky phenotype of *ura5*$^-$ mutants.

A substantial proportion (e.g., 30-50%) of 5-FOA-resistant mutants are URA$^+$ possibly due to mutations in the salvage pathway which prevent uptake of 5-FOA. Three genes, *FUR4*, *FCY2* and *FUI1*, are now known to encode permeases for uptake of uracil, cytosine and uridine, respectively (Jund et al., 1988; Weber et al., 1990; Kern et al., 1990), but a gene controlling specific uptake of orotate has not been described. Another explanation for FOA^R URA$^+$ mutants is that they are unable to induce the pyrimidine salvage pathway. A regulatory role for the *FOR1* gene (identified and so named by the isolation of mutants that were resistant to 5-FOA) is suggested by their reduced incorporation of radiolabelled orotic acid, adenine and notably cytosine into nucleic acids (Jund and Lacroute, 1972).

Spontaneous mutation spectra for the *URA3* gene exhibit an even distribution of mutations throughout the open reading frame with few, if any, hot spots (Lee et al., 1988; this study).

C. Mitotic recombination

The diploid strain, D7, was constructed by Zimmermann et al. (1975) for simultaneous detection of interchromosomal recombination and reverse mutation. The heteroalleles at *ADE2* permit an assessment of both mitotic crossing over and gene conversion, since homozygosis of either allele yields distinguishable red or pink colonies. Colonies with red and pink sectors derive from crossing over during the G2-phase, and all other sectored or unsectored colonies arise from conversion events during the G1-phase. The heteroalleles at *TRP5* provide an additional assessment of gene conversion.

Strain D7-144 is a sporulation-deficient derivative of strain D7 designed to exclude the induction of meiosis (and meiotic recombination) by recombinagenic agents. This strain thus allows for evaluation of recombination resulting from mitotic DNA repair separately from the more frequent exchange that occurs during meiosis (Mehta and von Borstel, 1985).

CHAPTER 2: MATERIALS AND METHODS

I. Strains

The genotypes of the strains of *Saccharomyces cerevisiae* used to assess mutation and recombination are tabulated in Table 1. The strains were stored at 4°C on YEPD-agar, from which colony isolates were checked routinely for their genetic properties. Frozen stocks were also kept at -70°C in YEPD supplemented with 10% glycerol. Isolation of cultures from frozen stocks was seldom necessary except for the DNA repair mutants, where it was desirable to minimize any deviation of spore genotypes through selection after meiosis.

II. Materials

A. Media

Stock solutions of amino acids and bases (at 100X conc) were autoclaved at 121°C for 15 min. or filter-sterilized (0.22 μm cellulose filter). The latter method was used for more critical concentrations.

YEPD (1% yeast extract, 2% peptone, 2% dextrose), synthetic complete (SC) and Mortimer complete (MC) media were used to grow overnight cultures and to assess viability in cell populations. SC consists of minimal medium (0.67% yeast nitrogen base—YNB, without amino acids and 2% dextrose) supplemented with amino acids (20 μg/ml L-arginine, L-histidine, L-methionine, L-tryptophan; 30 μg/ml L-lysine, L-isoleucine, L-tyrosine; 50 μg/ml L-leucine, L-threonine, L-phenylalanine; and 150 μg/ml L-valine) and bases (20 μg/ml adenine and uracil). MC is the same as SC but without valine, isoleucine, tyrosine and phenylalanine, and was suitable for growing strains without these requirements. Omission media (e.g., SC-his) were used to select for revertants in strains which carry amino acid requirements for the omitted component. Agar (1.5%) was added to the above recipes to prepare solid medium. Dextrose, YNB and agar were autoclaved in separate flasks, primarily to avoid loss of the gelling property of the agar. Amino acids and bases were added last, upon combining the autoclaved solutions.

TABLE 1: Yeast strains

Strain	Genotype	Source or reference
XV185-14C	*MAT*a *ade2-1 arg4-17 lys1-1 trp5-48 hom3-10 his1-7*	Shahin and von Borstel (1977)
ES55-6A	*MAT*a *ade2-1 arg4-17 ino1-13 trp5-48 ura3-52 leu2-3,112 his3-11,15* [Ylp-GL1 *URA3, LEU2, HIS3*]	Lee et al. (1988)
ES63-11A	*MAT*a *ade2-1 arg4-17 lys1-1 trp5-48 ura3Δ leu2-3,112 his3-11,15* [Ylp-GL1 *URA3,LEU2,HIS3*]	E.A. Savage
FL4761C-1A	*MAT*a *ura5-7 gal2*	Yeast Genetic Stock Centre
FL4761C-1B	*MATα ura5-7 gal2*	Yeast Genetic Stock Centre
ES58-15D	*MATα ade2-1 arg4-17 hom3-10 leu2-3,112* (*his1* or *his3*) *trp5-48 ura3Δ* (438 → 882)	E.A. Savage
ES59-16D	*MATα ade2-1 arg4-17 lys1-1 leu2-3,112* (*his1* or *his3*) *ura3Δ* (251 → 882)	E.A. Savage
GR450	*MATα ade2-1 arg4-17 lys1-1 trp5-48 ura3Δ* (189 → 643)	R.G. Ritzel
FLAC	*MATα ura3Δ* (-21 → 833)	F. Lacroute
D7-144	*MAT*a *ade2-40 trp5-12 ilv1-92* *MATα ade2-119 trp5-27 ilv1-92*	Mehta and von Borstel (1985)
HP159-2D	*ade2-1 arg4-17 lys1-1 trp5-48 hom3-10 his1-7 rad1-1*	P.J. Hastings
HP159-2B,2C	*ade2-1 arg4-17 lys1-1 trp5-48 hom3-10 his1-7 RAD1*	P.J. Hastings

HP160-4B		*ade2-1 arg4-17 lys1-1* *trp5-48 hom3-10 his1-7 rad2-2*	P.J. Hastings
HP160-4A		*ade2-1 arg4-17 lys1-1* *trp5-48 hom3-10 his1-7 RAD2*	P.J. Hastings
HP163-2C,2B		*ade2-1 arg4-17 lys1-1* *trp5-48 hom3-10 his1-7 rad6-1*	P.J. Hastings
HP163-2A,2D		*ade2-1 arg4-17 lys1-1* *trp5-48 hom3-10 his1-7 RAD6*	P.J. Hastings
HP166-3D		*ade2-1 arg4-17 lys1-1* *trp5-48 hom3-10 his1-7 rad18-2*	P.J. Hastings
HP166-3A,3B		*ade2-1 arg4-17 lys1-1* *trp5-48 hom3-10 his1-7 RAD18*	P.J. Hastings
HP167-2C,2D		*ade2-1 arg4-17 lys1-1* *trp5-48 hom3-10 his1-7 rad18-3*	P.J. Hastings
HP167-2A,2B		*ade2-1 arg4-17 lys1-1* *trp5-48 hom3-10 his1-7 RAD18*	P.J. Hastings
HP171-2B,2D		*ade2-1 arg4-17 lys1-1* *trp5-48 hom3-10 his1-7 rad52-1*	P.J. Hastings
HP186-3B,3D		*ade2-1 arg4-17 lys1-1* *trp5-48 hom3-10 his1-7 rad10-1*	P.J. Hastings
HP186-3A		*ade2-1 arg4-17 lys1-1* *trp5-48 hom3-10 his1-7 RAD10*	P.J. Hastings
QW4-1B,1D	*MATa*	*ade2-1 arg4-17 lys1-1* *hom3-10 rev3-15*	Q. Wang
QW4-1A,1C	*MATα*	*ade2-1 arg4-1 lys1-1* *hom3-10 REV3*	Q. Wang
QW8-11C		*ade2-1 arg4-17 lys1-1* *hom3-10 rev3Δ*	Q. Wang
QW8-16C		*ade2-1 arg4-17 lys1-1* *hom3-10 REV3*	Q. Wang

SC medium containing 7.3 mM 5-FOA (5-fluoroorotic acid monohydrate, PCR Inc. Organic Chemicals Division, SCM Corporation, Gainsville, Florida) was adjusted to pH 3.8 with K_2HPO_4. For preparation of 600 ml of SC+FOA agar medium (30-35 plates), dextrose (2%), agar (1.5%), YNB without amino acids (0.67%) and K_2HPO_4 (7.3 mM) were autoclaved in separate flasks. The first three components were combined while hot, to which 0.84 g 5-FOA was added and stirred with a magnetic bar until dissolved. Amino acids and nitrogenous bases were added from sterile stock solutions to the molten agar, combined with the dextrose-YNB-K_2HPO_4-FOA, and poured into plates. The volume of agar per plate (~15-17 ml) was somewhat less than normal to maximize the use of 5-FOA which is a fairly expensive reagent. Boeke et al. (1984) use less FOA in their recipe.

III. Reversion assays for base substitutions and frameshift mutations

An overnight culture (30°C) of the yeast strain was prepared by inoculation of cells from a pink colony that has been isolated recently on YEPD-agar. Cells from the overnight suspension were reinoculated into a larger volume (e.g., 50-200 ml) of fresh medium. A haemocytometer was used to quantify the cells in the inoculum so that a logarithmic phase suspension with not more than 5×10^7 cfu/ml could be obtained several hours after reinoculation. Most haploid strains in this study have a generation time of 90 min.

Logarithmic phase cells obtained after the predetermined growth period were counted with a haemocytometer, harvested in a Sorvall centrifuge (5,000 rpm, 2-5 min) and resuspended in 0.05-0.1X the original volume with H_2O. The cell suspension was subdivided to set apart the untreated cells, as a control, from the cells which were subjected to osmotic shock. The latter were diluted with one volume of salt or sugar solution, and the control suspension with one volume of H_2O or sodium phosphate buffer (0.2 M, pH 7.0). Thus, for treatment of cells in 2 M NaCl, a 4 M solution was diluted 2-fold. The cells were incubated in a rotary bath shaker at 30°C from which aliquots were removed at timed intervals to determine survival and reversion yield. In experiments which utilized stationary phase cells, a logarithmic phase population was maximized in G_0-phase by resuspending the cells in 0.1 M phosphate buffer and incubating at 30°C overnight. The cells were tested for survival and mutability in hypertonic conditions the following day, as described for logarithmic cells.

For estimation of the concentration of cells and plating efficiency before treatment, and survival of cells after treatment in hypertonic solutions, the cells were diluted to obtain approximately 200 cells per plate (containing SC or YEPD). Survival data obtained from pilot tests were used to gauge the survival in subsequent tests so that dilution factors for cells exposed to longer treatment in hypertonic solutions were lowered to raise the number of scorable colonies per plate. This was necessary for treatment times that reduced the viability by more than 2 orders of magnitude.

IV. Assessment of Mutations at the *URA3* Gene

A. Selection of 5-fluoroorotate-resistant colonies

FOA-resistant colonies were obtained on SC+FOA plates or, in the case of strains ES55-6A and ES63-11A, SC-leu+FOA plates. Resistance to 5-FOA, as noted earlier, is conferred by forward mutations in at least 3 genes: *URA5* and *URA3*, which encode enzymes for the final two steps in *de novo* biosynthesis of uracil (Jund and Lacroute, 1972; Boeke et al., 1984; Lee et al., 1988), and *FOR1*, which appears to function in a regulatory capacity (Jund and Lacroute, 1972). Presumably, *for1* mutants are not auxotrophic for uracil, thus accounting for the majority of URA^+ colonies isolated on SC+FOA. Since only a few mutations at *URA5* are selectable on SC+FOA, most of the ura^- mutations are at the *URA3* gene.

Prior to plating on SC+FOA, the cells were diluted in SC-ura and grown for ~3 generations (6-7 hours), as monitored by cell counts taken during the growth period. For samples that were previously growing in SC supplemented with NaCl, the cells were diluted in SC-ura without NaCl. The growth period for these cells in SC-ura was usually in the range of 8-10 hours. Following growth in SC-ura, the cells were plated directly on to SC+FOA agar medium and incubated at 30°C. Most FOA^R colonies arose within 5 days after plating; however, a few colonies appeared between 5 and 10 days. In this respect, it may be pertinent to consider that freshly prepared plates containing the monohydrate form of 5-FOA show very little indication of residual growth of cells on the plate and few, if any, colonies beyond 5 days.

B. Complementation analysis

FOA^R colonies were transferred from SC+FOA to YEPD plates and characterized by complementation. The isolates were repatched to a fresh YEPD plate and grown for 6-10 hours. At the same time, overnight cultures of *ura3*$^-$ and *ura5*$^-$ strains of the opposite mating type (*MATα*) were inoculated. The ura^- tester strains were centrifuged (5000 rpm) in Eppendorf tubes, resuspended in H_2O and spread as a lawn on SC-ura plates (0.5 ml per plate). The agar was adequately dried beforehand so as to soak up this volume of liquid suspension. SC-ura plates, thus prepared, were replica plated with the FOA^R isolates on YEPD using a separate velvet for each transfer. Since identification of the *ura3*$^-$ mutations was of primary interest, complementation tests to identify the *ura5*$^-$ colonies were often not included; moreover, mutations at the *URA5* gene generally comprise only a small fraction of the total ura^- mutations selected on SC+FOA. Isolates which complemented both tester strains were considered to be URA^+; and those that did not grow were allelic with either *URA3* or *URA5*.

C. Deletion mapping

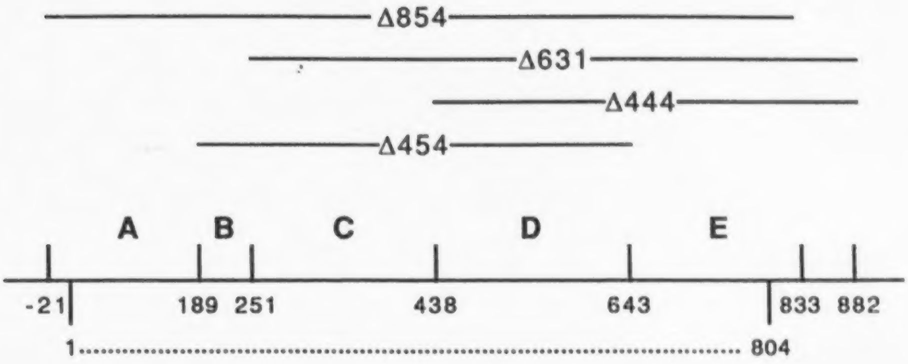
Ura3$^-$ mutants identified by complementation were mapped by crossing the isolates to each of four *MATα* tester strains which carry different deletions of the coding region of the *URA3* gene (Figure 2). Several modifications to the general protocol were developed to optimize recovery of recombinant colonies on SC-ura plates. Spreading the diploid cells in liquid suspension (cf. replica plating) provided a more uniform distribution of the cells on SC-ura plates for induction of mitotic recombination by uv irradiation. Diploids were formed by mixing the tester strains (prepared beforehand as a lawn of cells on YEPD plates) with the $\mathit{ura3}^-$ isolates in a 2 cm^2 patch on YEPD-agar using sterile splints. Diploid strains outgrow the haploid cells and are ready for inoculation to liquid YEPD in separate tubes within 6 hours of crossing the haploid derivatives. Overnight cultures of the diploid cells were harvested (5000 rpm) in Eppendorf tubes, diluted 2-fold or more in H_2O and aliquoted (0.01-0.02 ml of cell suspension) to 2 cm^2 patches on SC-ura plates.

The plates were irradiated with uv (254 nm) from a GE low pressure mercury lamp (General Electric G30T8 30W germicidal lamp) at a distance of 50 cm (45 sec per plate) to induce mitotic recombination. The intensity of uv light, as measured by a calibrated uv dosimeter (from Dr. Raymond Latarjet, Laboratoire Pasteur de L'Institute du Radium, Paris) was ~0.79 J m^{-2} sec^{-1}, thus providing a fluence of 35 - 36 J/m^2 in 45 seconds. Since 31 - 32 J/m^2 resulted in a surviving fraction of ~0.50, as determined in diploids crossed with the haploid strain ES63-11A (data not shown), a fluence close to 40 J/m^2 gave the maximum yield of recombinants per plate. The yield of recombinants was reduced if too many cells were plated per patch. Hence, replica plating from YEPD plates directly to SC-ura generally caused transfer of an excessive amount of cells and, in turn, too much background growth which obscured the appearance of recombinant colonies.

The plates were wrapped in foil to preclude photoreactivation and incubated (30°C) for 2 to 4 days. Colonies which grew after this time were scored as shown in Figure 2, where "+" represents a clear indication of recombination with the deletion strain. Recombination (due to mitotic conversion) was distinguishable from reversion by comparing the number of colonies induced by uv for the various diploid strains to the diploid that carries a deletion of the entire coding region of the *URA3* gene (Δ854, -21→833). This strain acted as a control for assessment of the revertability of the $\mathit{ura3}^-$ mutations as selected on SC+FOA, because few, if any, of the mutations occur in the promoter or tail region which are the only regions where recombination can occur for this strain. The number of recombinant colonies obtained per patch is a function of the location of the mutation in the $\mathit{ura3}^-$ (*MATa*) isolate

FIGURE 2:

Deletion tester strains used to map *ura3*$^-$ mutations. The four deletions (Δ854, Δ631, Δ444 and Δ454) in the tester strains (FLAC, ES59-16D, ES58-15D and GR450) used for fine structure mapping of *ura3*$^-$ mutations from isolates selected on SC+FOA are diagrammed above, which shows the regions (A → E) available for mitotic conversion in the table below. The open reading frame of the *URA3* gene is depicted by the dotted line.


Δ854
Δ631
Δ444
Δ454
A
B
C
D
E
-21
189
251
438
643
833
882
1
804

Mutation in gene region	Recombination with			
	Δ444	Δ631	Δ454	Δ854
A	+	+	+	–
B	+	+	–	–
C	+	–	–	–
D	–	–	–	–
E	–	–	+	–
Promoter/Tail	+	+	+	+

relative to the endpoints of the deletion in the *MATα* tester strains. In general, the number of recombinant colonies per patch was in the range of 100 to 200, compared to 0 to 10 revertants. *Ura3*$^-$ mutations that approach a deletion endpoint can still produce a significant increase in the number of recombinants scored, as revealed by two nonrevertable *ura3*$^-$ mutations positioned 11 nucleotides upstream from a deletion endpoint (commencing at nucleotide 251 in Δ631). The diploid strain bearing the Δ631 deletion produced 19 recombinants (mean of 3 measurements) for ~1 - 2 X 10^6 plated cells. Another mutation, this time revertable, positioned 20 nucleotides upstream from the same endpoint, produced 38 more colonies (mean of 2 measurements) than the number of revertant colonies by the same plating conditions.

D. Extraction of genomic DNA

Extraction of genomic DNA from strains ES55-6A and ES63-11A was carried out according to Davis et al. (1980) with some modifications. These strains carry an integrating plasmid, YIp-GL1 (pUC18 with yeast genes *HIS3*, *LEU2* and *URA3*) which, upon extraction from the yeast cells, was used to transform *E. coli* to ampicillin resistance. Total yeast DNA (~20 μg from a 1.5 ml preparation) was digested to completion with *Bam*H1 in a volume of 50 μl (10 μg DNA/10 μl TE pH 8.0, 5 μl 10X reaction 3 buffer, 10 units *Bam*H1), then extracted with PCI (70% phenol: 30% chloroform: 1% isoamyl alcohol). The DNA was precipitated in ethanol, dried and ligated (15°C, 6h or overnight) in a 50 μl reaction mixture (0.5 units ligase, 5 μl 10X ligase buffer). Preparation of competent cells of *E. coli* strain HB101 for transformation was according to Maniatis et al. (1982). Small-scale preparations of plasmid DNA ("mini-preps") from individual transformant colonies were conducted by the alkaline lysis method of Birnboim and Doly (1979). This generally provided enough DNA for sequencing. Enzymes for restriction or modification of DNA were purchased from either Pharmacia P-L Biochemicals or Bethesda Research Laboratories and used as recommended by the suppliers.

With the development of PCR (polymerase chain reaction), it became possible to bypass the transformation and amplification steps in *E. coli*;

instead, the integrating plasmid (for strain ES63-11A) or the genomic copy of *URA3* in any strain of yeast could be amplified directly from an extract of genomic DNA. Ultimately, the protocol was simplified even further by extracting DNA directly from yeast colonies.

Using a disposable pipette tip, cells from a yeast colony were transferred to 200 µl of freshly prepared sorbitol/EDTA solution (1 M sorbitol, 20 mM EDTA, 10 µl/ml β-mercaptoethanol, and 2 mg/ml zymolyase 20T from ICN #320921), then incubated for 5 min at 37°C. Extraction of DNA was facilitated by adding 100 µl PCI, vortexing, then heating this mixture for 5 min at 60°C. The samples were microfuged (14,000 g, 1 min) and the aqueous phase was transferred to new Eppendorf tubes. The DNA was purified with a GeneClean II kit (BIO 101 #3106), by first adding 300 µl 6 M NaI and 3 µl of well suspended glass milk and allowing at least 5 min (22°C) for uptake of DNA into the glass beads. The glass milk was spun down (5-10 sec) and rinsed twice with 350 µl New Wash solution (in kit) at -20°C, using an aspirator to draw off the supernatant after each spin. After the second rinse, liquid drops inside the tube were thoroughly removed and the pellet was set aside to dry for 2-30 min (22°C). The DNA was extracted from the glass milk into 21 µl distilled H_2O by mixing the suspension with a pipette tip. The samples were then heated to 55°C for 5 min, spun down (2 min) and the aqueous phase (20 µl) was transferred to a new tube.

All of the preparations of genomic DNA described above were stored at -20°C.

E. PCR amplification of DNA

For PCR amplification of plasmid DNA obtained from a small-scale preparation of genomic DNA (dissolved in 50 µl TE, pH 7.4), 5 µl (~1 µg DNA) was mixed with 25 µl 1.2X PCR mix and 0.5 µl (2 units) *Taq* polymerase (BioCan Scientific MB3024 or Promega Sci. PRM 1861). For DNA prepared directly from colonies (in 20 µl H_2O) 2 µl was mixed with 15 µl 1.2X PCR mix. The final concentration of PCR mix was 65 mM Tris (pH 9.0), 5 mM $MgCl_2$, 15 mM NH_4SO_4, 5-10 mM β-mercaptoethanol, 0.1 mg/ml bovine serum albumin, 0.5 mM for each of the 4 deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP), and 2.5 µg/ml for each of the two flanking *URA3* primers. Once the *Taq* polymerase was combined with PCR mix and distributed to 500 μl Eppendorf tubes, the DNA samples were added and the aqueous reaction mixture was overlain with a drop of parafin oil to reduce evaporation.

The following oligonucleotides (16-21 nucleotides in length), synthesized by Dr. Ken Roy (Dept. Microbiol., Univ. Alberta), were used to prime the polymerization reactions. Primers at the 5' end of the *URA3* gene, all of which correspond to the transcribing strand, were α (-156 → -136), FL (-49 → -32), and A (-22 → -7). Primers for extension of the template strand from the 3' end of the gene were either β (883 → 903) or pR2 (186 → 201). For PCR amplification of plasmid DNA from strain ES63-11A which carries a deletion of the genomic copy of the *URA3* gene from -21 to 833, primers A and β or α and pR2 were used to avoid amplification of the undeleted flanking regions of the gene. For all other strains, primers α and β or FL and β were generally used.

PCR was conducted in a temperature-programmable cycler which rapidly modifies the temperature of the sample chamber at programmed intervals. For amplification of ~1 kb of the *URA3* gene the following temperature program worked well for both the air temperature-controlled cycler (Thermal Reactor TR96, Tyler Research Instruments Corp.) or a water bath cycler (Autogene II, Grant Scientific). The samples were initially denatured for 5 min at 96°C; thereafter, the following steps were repeated through 30 cycles: primer annealing to the template, 40 sec at 60°C; elongation, 1 to 3.5 min at 76°C; and redenaturation, 10 sec at 93°C. The entire program, including ramping time to reach the setpoint temperatures, was generally in the range of 2.5-3.5 hours.

Following PCR, the amplified DNA was isolated by electrophoresis. The aqueous phase containing the amplified DNA was transferred to clean tubes, to which 0.1 volume of 10X tracking dye (0.25% bromophenol blue or Ficoll-400 in 50% glycerol from Fisher Scientific) was added. The entire sample (~18 or 30 μl) was loaded to a 0.8% agarose gel prepared in 1X TAE buffer (40 mM Tris, 1 mM EDTA, pH 8.2) and run at 80-100 volts for 30 min. The bands, as visualized by fluorescence of ethidium bromide (0.5 mg/ml of gelled agarose), were cut out and the gel slice transferred to clean tubes. The DNA was extracted from the agarose with a GeneClean II kit. Assuming a density of ~1, the gel slices were weighed and three volumes (~400 µl) of 6 M NaI were added. The samples were incubated for 5 min (55 °C) and vortexed to ensure that the gel was completely dissolved in the salt solution. After setting the samples at room temperature to cool, 3 µl of well suspended glass milk was added, spun down (10 sec), washed (as described above), and eluted with 14 µl distilled H_2O. The samples in H_2O were then ready for DNA sequencing.

F. DNA sequencing of the *URA3* gene

1. Preparation of polyacrylamide gels

Most sequencing gels were prepared with 5% acrylamide. A one liter recipe consisted of 480 g urea, 47.5 g acrylamide (ICN or Boehringer Mannheim Corp.), and 2.5 g bis-acrylamide (BRL), brought to 900 ml with H_2O. The premix solution was deionized for at least 30 min with an ion exchange resin (Bio-Rad mixed bed resin, AG 501-X8, 20-50 mesh), filtered through a Buchner funnel, and topped to 1 L with 100 ml of 10X TBE buffer. Premix acrylamide was stored in a brown bottle at 4°C.

A 5% polyacrylamide gel was generally prepared on the previous day, although 1 or 2 hours is adequate time to allow for polymerization of the acrylamide. The gel was poured between two glass plates (20 X 50 cm) separated by 0.4 mm Mylar spacers. The plates were cleaned, siliconized (5% dimethyldichlorosilane in heptane) and dried prior to being clamped together. Crosslinking of the acrylamide was induced by adding 40-50 µl of 25% ammonium persulfate (Fisher Scientific) and 40-50 µl of TEMED (*N,N,N',N'*-tetramethylethylene-diamine, Bio-Rad seq. grade), whereupon the acrylamide was poured immediately between the glass plates. The top spacer was inserted last, extending ~3 mm below the edge of the top plate into the acrylamide.

2. Sequencing reactions

DNA sequencing was conducted by the dideoxy chain termination method (Sanger et al., 1977; Tabor and Richardson, 1987). A single primer (100 ng) in 1 µl was added to 12-13 µl of PCR-amplified template prepared from extracted colonies. For each sample, a suitable primer was chosen on the basis of deletion mapping of the *ura3⁻* mutations into 5 possible regions, as shown in Figure 2. The primers used for sequencing which correspond to these regions of the *URA3* gene were as follows: region A or B, pFL (-49 → -32), pA (-22 → -7) and p2 (186-201); region C, p2 and pB (282-298); region D, p4 (412-427); region E, pC (580-595) and pβ (883 → 903). After the primers were added, 3 μl 5X annealing mix (Reaction Buffer, USB Corp) was added (although better results were later obtained by leaving out the annealing mix, but $MnCl_2$ was then essential in the sequencing reaction mix described below; R. G. Ritzel, person. commun.). The samples were boiled in a water bath for 3-4 min, then promptly frozen in dry ice/ethanol (-70°C). A short spin (3-5 sec) in the microfuge was sufficient to draw down the condensate.

The samples were kept on ice, while 2.5 μl of each of 4 termination mixes (each containing small amounts of 1 of 4 dideoxynucleotide triphospates: ddATP, ddCTP, ddGTP, and ddTTP) were dispensed into Eppendorf tubes. A master mix for the polymerization reaction was prepared which contains the following components per sample: 1 μl dithiothreitol (0.1 M), 1 μl labelling mix (USB Corp.), 1 μl H_2O, 0.5 μl [α ^{35}S]dATP (5-6 μCi; Amersham Corp. - 600 Ci/mmole), 0.5 μl Mn^{2+} buffer (USB Corp.), 0.25 μl Sequenase® (~3 units; USB — 13 units/μl). Reaction mix was usually prepared for 16 samples, which can be accommodated by two 20 cm X 50 cm sequencing gels. To carry out the polymerization reaction, the stop tubes (i.e., containing termination mix) were transferred to a 37°C temperature-controlled block, and at the same time ~4 μl of master mix was aliquoted to the samples (i.e., template-primer mix on ice), allowing for brief thawing of the mix. The components were mixed with a pipettor, and then 4 to 4.5 μl of this elongation reaction mix was aliquoted as a small drop to the inside rim of the stop tubes. After a reaction time of not more than 4 minutes (20°C), the chain elongation was stopped by spinning down the droplets (10 seconds in a SpeedVac) into the 37°C termination mix. The tubes were returned to the 37°C block for a further 3 to 5 minutes, following which 5 μl gel loading buffer (0.1 mM EDTA, 0.1% xylene cyanol FF, and

0.1% bromophenol blue in formamide) was added. The samples were spun down and boiled for 3 minutes, at which time they were ready for loading to a polyacrylamide gel.

3. Acrylamide gel electrophoresis

After removing clamps and top and bottom spacers, the acrylamide gel was fastened to a vertical polyacrylamide gel electrophoresis unit (Tyler Research Instruments, Edmonton). The reservoirs were filled with buffer (1X TBE), and a 32-well Mylar sharkstooth comb was inserted into the top space so that the teeth sealed against the well bottom. The wells were flushed several times with a Pasteur pipette to remove excess urea just before loading the samples. Using a Hamilton syringe (10 µl capacity), 2 - 2.5 µl of each reaction mix (A, C, G, T) was dispensed in this order into the slots.

The gel was run at 50 W of constant power from a Bio-Rad power supply (Model 3000/300) for 2 to 2.5 hours, as monitored by the advance of the blue tracking dye (i.e., bromophenol). The power was stopped just before the dye ran into the lower reservoir. Following electrophoresis, the gel was blotted to a pre-cut sheet of Whatman 3MM chromatography paper, covered with plastic wrap and dried for 2 hours at 80°C on a gel drier (Bio-Rad Laboratories, Model 583). The gel was checked for approximate radioactivity with a scintillation counter. A rate of 5 to 10 counts per second (or higher) was normally obtained.

Autoradiography was conducted using Fuji X-ray film, allowing for 2 to 3 days of exposure in an X-ray cassette (Kodak X-Omatic). The film was developed with Kodak developer (3 min), followed by acetic acid stop solution (60 sec with agitation) and Kodak fixer (3 to 4 min). After a rinse with cold water (5 min) the DNA sequence was read from the autoradiograph.

The DNA sequence of the *URA3* gene and primers used for routine sequencing of the gene are shown in Figure 3.

FIGURE 3:

Nucleotide sequence of the *URA3* gene. The DNA sequence for the nontranscribed strand (5' → 3') is represented in groups of 10 nucleotides, numbered according to the open reading frame which commences with an **ATG** codon at +1 and terminates at a **TAA** codon at +802. The sequence encodes an enzyme, OMP decarboxylase, with 267 amino acids (M_r = 27,500). Among the strains used for sequencing in this laboratory, XV185-14C exhibits 3 silent polymorphisms at -81 (**A**→G), 411 (**A**→G) and 478 (T→G), which are not present in strain ES63-11A. Rose et al., (1984) also found these polymorphisms in the original 2 wild type strains (+D4 and FL100) sequenced. Primers used to sequence *ura3*$^-$ mutants are underlined for oligonucleotides which correspond to the nontranscribed strand. A line above the sequence (e.g., pβ) represents a primer from the transcribed strand which is complementary to the marked sequence.

```
     AGCT   TCAATTCAAT   TCATCATTTT   TTTTTTATTC   TTTTTTTTGA
-180
TTTCGGTTTC   TTTGAAATTT   TTTTGATTCG   GTAATCTCCG   AACAGAAGGA
                                            pα
-130
AGAACGAAGG   AAGGAGCACA   GACTTAGATT   GGTATATATA   CGCATATGTA
-80
GTGTTGAAGA   AACATGAAAT   TGCCCAGTAT   TCTTAACCCA   ACTGCACAGA
                                                   pFL
-30                                    1
ACAAAAACCT   GCAGGAAACG   AAGATAAATC   ATGTCGAAAG   CTACATATAA
                 pA
21
GGAACGTGCT   GCTACTCATC   CTAGTCCTGT   TGCTGCCAAG   CTATTTAATA
71
TCATGCACGA   AAAGCAAACA   AACTTGTGTG   CTTCATTGGA   TGTTCGTACC
121
ACCAAGGAAT   TACTGGAGTT   AGTTGAAGCA   TTAGGTCCCA   AAATTTGTTT
171
ACTAAAAACA   CATGTGGATA   TCTTGACTGA   TTTTTCCATG   GAGGGCACAG
                             p2
221
TTAAGCCGCT   AAAGGCATTA   TCCGCCAAGT   ACAATTTTTT   ACTCTTCGAA
271
GACAGAAAAT   TTGCTGACAT   TGGTAATACA   GTCAAATTGC   AGTACTCTGC
                        pB
321
GGGTGTATAC   AGAATAGCAG   AATGGGCAGA   CATTACGAAT   GCACACGGTG
371
TGGTGGGCCC   AGGTATTGTT   AGCGGTTTGA   AGCAGGCGGC   AGAAGAAGTA
                                                          p4
421
ACAAAGGAAC   CTAGAGGCCT   TTTGATGTTA   GCAGAATTGT   CATGCAAGGG
471
CTCCCTATCT   ACTGGAGAAT   ATACTAAGGG   TACTGTTGAC   ATTGCGAAGA
521
GCGACAAAGA   TTTTGTTATC   GGCTTTATTG   CTCAAAGAGA   CATGGGTGGA
571
AGAGATGAAG   GTTACGATTG   GTTGATTATG   ACACCCGGTG   TGGGTTTAGA
621
TGACAAGGGA   GACGCATTGG   GTCAACAGTA   TAGAACCGTG   GATGATGTGG
                               pC
671
TCTCTACAGG   ATCTGACATT   ATTATTGTTG   GAAGAGGACT   ATTTGCAAAG
721
GGAAGGGATG   CTAAGGTAGA   GGGTGAACGT   TACAGAAAAG   CAGGCTGGGA
771                                    801
AGCATATTTG   AGAAGATGCG   GCCAGCAAAA   CTAAAAAACT   GTATTATAAG
821
TAAATGCATG   TATACTAAAC   TCACAAATTA   GAGCTTCAAT   TTAATTATAT
871                           pβ
CAGTTATTAC   CCGGGAATCT   CGGTCGTAAT   GATTTTTATA   ATGACGAAAA
921
AAAAAAAATT   GGAAAGAAAA   AGCTT
```

V. Measurement of Spontaneous Reversion and Forward Mutation Rates

A. Compartmentalization test

An overnight culture (30°C) of a given yeast strain for assessment of reversion rate in the compartmentalization test was diluted in H_2O and the cells were counted with a haemocytometer. The concentration was adjusted so that a 0.25 to 1.0 ml inoculum for 600 ml of limiting medium contained ~1.2 - 2.4 X 10^6 cells. Optimization of the limiting concentration of amino acids in the medium was based on pilot experiments in which 30 to 70% of the total compartments were without colonies. These concentrations are specified in the tables in chapter 3 (see Tables 11-13, 15 and 30) for each reversion system used.

Once inoculated with 2000 - 4000 cells/ml, the limiting medium was dispensed in 1 ml aliquots to 552 compartments in 23 cell culture trays (24 wells per tray, Falcon Plastics) with a Brewer automatic pipetting machine, model No. 40 (Baltimore Biological Laboratory). To maintain a uniform cell suspension throughout delivery, the medium was stirred continuously with a magnetic stirring bar. The frequency of revertants contributed by the inoculum to the limiting medium was estimated by plating the cells from the original suspension to an appropriate omission medium. Confirmation of cell viability in the inoculum was also determined by plating ~200 cfu to YEPD plates at the time the medium was dispensed into trays.

After distribution of the suspension into compartments, the trays were sealed in a plastic bag to minimize evaporation and incubated at 26°C for at least 10 days. In a limiting medium, the cells grow to saturation within 24 - 48 hours. The revertants continue to divide beyond this point and form colonies that begin to appear within 3 days. After 10 to 12 days, the average number of cells per compartment was determined by taking cell counts from wells without colonies, usually ~20 measurements (1 for each culture tray). Assessment of reversion rates for a hypertonic medium sometimes required a prolonged growth (e.g., 20 - 25 days for 0.30 M saccharin), owing to longer generation times. Both generation time and cell viability were monitored prior to the appearance of colonies. The kinetics of colony formation following growth of the cells to saturation, and other details concerning this procedure are described by von Borstel (1978).

The rate of reversion, M, was determined from the following expression:

$$M = \frac{m - m_b}{2C}$$

where C and m denote the average number of cells and revertants per compartment, respectively; and m_b equals background spontaneous reversion frequency. A value of m can be obtained by the P(0) method (where $P_0 = e^{-m}$) or from assessment of the number of compartments bearing one or more revertants. A representative set of data obtained for strain HP163 (*rad6-1*) in Appendix 1 indicates the close conformity of the probability of reversion to a Poisson distribution.

B. Luria-Delbrück fluctuation tests

An overnight culture of the yeast strain was prepared in the same growth medium as that to which the cells were inoculated for the Luria-Delbrück fluctuation test. For one test, 50 tubes (each containing 5.0 ml of SC medium) were inoculated with 2.5 to 7.5 X 10^3 cells in 0.1 ml (~500 - 1500 cells/ml). The probability of inoculating one or more of the tubes with a *ura3*$^-$ mutant, which is ~10-fold more likely than introducing a revertant, can be reduced by preparing the overnight culture in SC-ura and inoculating to SC. The occasional introduction of a revertant or forward mutant in the inoculum was easily recognized, however, by the appearance of 1000 - 4000 revertants per plate, or in the case of *URA3*, 100 - 400 FOA-resistant colonies per plate. These plates were excluded from the assessment of mutation rate.

The tubes were incubated at 30°C in a test tube incubator shaker (New Brunswick Scientific Co.) for 20 - 25 hours, allowing for ~12 - 15 generations (generation time, 90-100 min) to obtain a cell concentration in the range of 3 - 8 X 10^7 cfu/ml. Incubation beyond the logarithmic phase (> 1 X 10^8 cfu/ml) was avoided to minimize selection of revertants in the population. The cells were harvested in 1.5 ml plastic Eppendorf tubes and centrifuged (5000 rpm) to obtain a pellet with a diameter of ~0.5 - 1.5 mm. Using a plastic pipette tip on the end of a Pasteur pipette and a vacuum aspirator, the medium (supernatant) was removed. The samples were washed once and resuspended in 1 volume of 2% dextrose, then plated on SC-arg, .SC-his and SC-thr medium (8×10^6 - 2×10^7 cells/plate). At the same time, 0.40 ml from each tube was diluted 10-fold in 3.6 ml SC-ura and incubated (30°C) for 6 - 8 hours to allow for approximately 3 generations of growth. This was necessary to induce depletion of the cellular pool of OMP-decarboxylase in *ura3*$^-$ mutants prior to plating the cells on SC+FOA. Once the cells were plated on omission media and diluted in SC-ura, the remaining sample (100-200 µl) was stored at 4°C to prevent residual growth beyond the concentration at which the cells were plated. Hence, the cell concentration for 15 - 20 of these samples could be assessed by haemocytometer at a later time. After growth for 6 to 8 hours in SC-ura, these cells were plated on SC+FOA medium, using 0.20 - 0.30 ml of cell suspension per quarter-sector of agar medium.

The plates were incubated at 30°C inside plastic bags to reduce moisture loss over a period of 15 - 20 days. Omission plates required at least 15 days to obtain the maximum number of revertants, most of which arose within the first 3 days. FOA-resistant colonies were counted 10 days after plating.

Residual growth on omission media was determined generally within 7 days after plating, before the cells begin to deteriorate. Two different methods proved satisfactory. In the first method, cells were extracted from the agar surface of plates without colonies. The agar was removed from the plate with a scalpel or flat knife to a calibrated flask, taking care to avoid contacting the area which bears the cells. The volume was brought to 100 ml and the agar melted by heating for 15 sec or more in a microwave oven. The cell concentration, as determined by haemocytometer, was then compared to the number of cells plated, based on the concentration and volume of cells plated and assuming a plating efficiency of 0.826 ± 0.0826 (23 measurements). If the residual growth was not greater than 2, a second method for assessing residual growth was possible. This involved taking cell counts by direct examination of the agar surface under the microscope and comparing the number of cells per cfu both before and after plating. The methods gave comparable results if conducted within 7 days and if the cell density per plate was not too large to permit a significant amount of overlap between adjacent cfu.

Reversion rates and forward mutation rates were calculated by 3 different methods, all of which provide an estimate of m (the average number of mutational events per culture) based on the observed distribution of r (the observed number of colonies per culture) among N cultures. These methods were derived mathematically by Lea and Coulson (1949) who expounded upon the theory of Luria and Delbrück (1943) which proposed that the the acquisition of *mutations* conferring phage-resistance in bacteria grown in parallel cultures follows a Poisson distribution, in contrast to the number of resistant cells (i.e., *mutants*) appearing in the bacterial population following growth.

The P(0) method estimates a value of m from the proportion of plates without colonies, where $P(0) = N(0) / N = e^{-m}$ on the assumption that the number of plates without colonies, $N(0)$, is determined by the Poisson expression. The method of the median uses an experimentally determined value of the median number of colonies per plate, r_o, to obtain an estimate of m, where $r_o/m - \log m = 1.24$ for a derived variate showing a normal distribution around a median of 0 (Lea and Coulson, 1949). The method of maximum likelihood (MML) is based on a probability distribution for N parallel cultures having r mutants per culture where,

$$0 = \sum_{i=1}^{N} \frac{t_r - P_r}{P_r}$$

i denoting the ith culture ($i = 1, 2, \ldots, N$) of N cultures in total and P_r represents the probability that a culture has r mutants ($r = 0, 1, \ldots 64.$). The derivation of P_r and t_r requires several functions and is presented elsewhere (Lea and Coulson, 1949; Koziol, 1991).

A comparison of the mutation rates as calculated by these different methods is provided in Appendix 2. The rate of reversion or forward mutation per cell per generation, M, is given by the formula $M = m/2CRve$, where C = the average cell concentration per culture, R = residual growth factor, v = volume of cells plated, and e = plating efficiency. For cells that underwent 6 - 8 hours of growth to deplete OMP decarboxylase, C is based on the concentration of cells prior to growth in SC-ura (i.e., diluted 10-fold) on the premise that newly formed *ura3*$^-$ mutants must overcome a phenotypic lag to be recovered as colonies on SC+FOA plates (Lee et al., 1988). The data in Appendix 2 show that rates based on P(0) values > 0.40 conform closely to those determined by MML; however, the precision of estimates of m and thus M, calculated by either method, declines as the value of m becomes smaller. This is exemplified by the 3 estimates of M for *URA3* → *ura3*$^-$ determined by the P(0) method from independent experiments ($M = 2.2 \pm 0.67 \times 10^{-7}$, Appendix 2) with P(0) values in the range of 0.68 to 0.76. The corresponding values calculated by MML from the same data exhibit a similar variance ($M = 2.0 \pm 0.57 \times 10^{-7}$, Appendix 2). On the other hand, a more precise estimate of M is obtained when the value of m exceeds say 1.2 (i.e., for P(0) < 0.30) when calculated by either the method of the median or MML. Thus, on the assumption that the presence of NaCl in the SC medium has no effect on the overall reversion rate at *his1-7*, the rates as calculated from 5 sets of data by the 3 different methods are as follows: $7.3 \pm 1.5 \times 10^{-7}$ (P_0), $5.1 \pm 1.1 \times 10^{-7}$ (median), and $6.0 \pm 0.65 \times 10^{-7}$ (MML).

C. Measurement of reversion rates from a single Petri plate

The Lassie test, as described by von Borstel (1978), was adapted so that reversion rates might be measured from a single plate of dividing cells. The principle is similar to that of the compartmentalization test in which growth of the yeast cells is constrained by reducing the concentration of an auxotrophic requirement in the medium. In the case of the compartmentalization test, the growth-limiting concentration was adjusted so that a P(0) method could be used to calculate a rate. For the Lassie test, the average number of revertant colonies per plate (a single compartment), as determined by the area of agar to which cells were applied and the growth-limiting concentration, was generally in the range of 50 - 150 for a nonmutator (i.e., DNA repair proficient) strain of yeast.

Based on these guidelines, the measurement of reversion rates at *arg4-17* was conducted using plates containing SC-agar medium with a reduced concentration of arginine (5 or 10 μg/ml), so as to limit the growth of the plated cells, and adenine (10 or 20 μg/ml, respectively), to elicit red pigmentation among ARG$^+$ colonies that are not suppressible at *ade2-1*. All other supplements to the SC medium were at 4-fold normal concentrations, to avoid the possibility of creating a dual requirement.

Single colony isolates of various yeast strains, including DNA repair-deficient mutants, were inoculated into YEPD broth and grown overnight (30°C). The cells were harvested and diluted 10-fold in H_2O. For each sample, 0.20 - 0.30 ml of sample was spread on to two sets of agar plates, consisting of SC medium without arginine and 5 μg/ml adenine (i.e., in one set) or containing the aforementioned growth-limiting concentrations of arginine and adenine (i.e., in the second set). The omission medium was used to measure the background level of revertants before cell division. The procedure, as described, dispensed ~2 to 6 X 10^6 cells per plate, allowing for 6 - 9 generations of growth on plates containing a small amount of arginine, but less than 2 generations (i.e., due to residual growth) on the omission medium.

The plates were evaluated for total number of colonies and number of cells per plate after 17 - 18 days. The latter was determined by coring a 1.3 cm diameter agar plug from an area of the plate in which cell growth occurred without colonies. The plug was placed in to a test tube, to which 10 ml of H_2O was added, and melted with gradual heating in a microwave oven. The cells thus released from the agar into suspension were counted by haemocytometer. The number of cells per plate, C, was determined by the formula $C = nHVr_1^2/r_2^2$, where n = number of cells counted (per 0.1 mm^3 grid on a haemocytometer), H = 10^4 (per ml), V = 10 (ml per tube), r_1 = radius of the circle of cellular growth on the plate, and r_2 = radius of the circular agar plug. Measurement of C by this method was calibrated by testing the recovery of cells from the agar surface immediately after plating cells and at later intervals.

Mutation rates, M, were calculated by the formula $M = m/2C$, where m and C represent the average or the total number of revertant colonies and cells per plate, respectively, from 3 or more experiments. The effect of NaCl upon mutation rates was determined by supplementing the limiting SC medium with 0.30 M NaCl.

CHAPTER 3: RESULTS

I. Genetic Effects of Osmotic Shock in *Saccharomyces cerevisiae*

A. Mutagenesis in logarithmic phase haploid cells

Saccharomyces cerevisiae, though not a xerotolerant species of yeast, can undergo cell division in a hypersaline growth medium supplemented with up to 2 molar NaCl. When tested for growth under these conditions, the haploid strain XV185-14C shows an increase in generation time from 1.8 hours in SC without salt (Figure 4) to 2.5 and 5.4 hours in SC supplemented with 1.0 and 2.0 M NaCl, respectively (Figures 4 and 5). Thus, in adapting to growth in hypertonic media yeast cells reduce the rate of cell division in proportion to the salinity.

The effects of hypertonicity on mutation in *Saccharomyces cerevisiae* were investigated by testing cells which have adapted to conditions of high salinity, as manifested by cell division, if a source of carbon and nitrogen is available in a hypertonic medium. These results are presented in part II of the results. Cells were also tested under shock conditions, in which the salinity of the aqueous environment of the cells is abruptly increased. The mutagenic and recombinagenic effects of osmotic shock are the subject of part I of this chapter.

Osmotic shock is both lethal and mutagenic to logarithmic phase cells of yeast. Survival data and reversion frequencies for logarithmic phase cells of yeast treated with 2 M NaCl or 2 M KCl are shown in Table 2. In these experiments the cells were pelleted by centrifugation to remove the growth medium and resuspended in either water or aqueous salt solution. The control data (distilled water) provides a measure of the spontaneous reversion frequencies for the three markers against which the induced effects can be compared. Both NaCl and KCl cause a significant reduction in survival of logarithmic phase cells. The effect is approximately the same, as indicated by the cell viability at 1.0 and 2.5 hours.

FIGURE 4:

Growth of *Saccharomyces cerevisiae* over 35 hours in SC medium supplemented with 1.0 or 2.0 M NaCl. Yeast cells from an overnight culture in YEPD of strain XV185-14C were inoculated to 50 ml of SC medium ± NaCl and incubated at 30°C in a rotary shaker. Cell counts, using a haemocytometer, were taken at 30-min. intervals. The growth curves corresponding to the different SC media are as follows: SC-NaCl (—Δ—), SC+1.0 M NaCl (—▲—), SC+2.0 M NaCl (—■—).

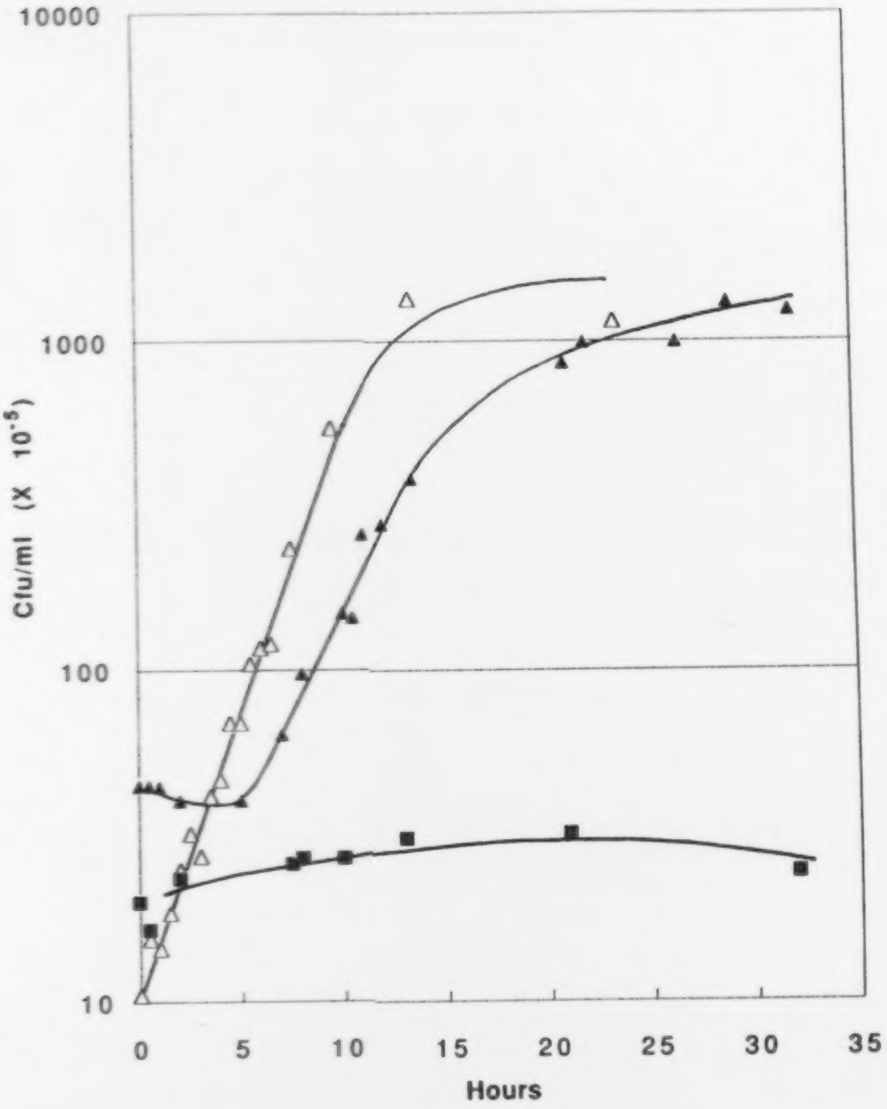

10000
1000
100
10
Cfu/ml (X 10^{-5})
0
5
10
15
20
25
30
35
Hours

FIGURE 5:

Growth of *Saccharomyces cerevisiae* over 350 hours in SC medium supplemented with 2.0 M NaCl. The time is extended in Figure 5 for yeast cells inoculated to SC + 2.0 M NaCl in Figure 4 (—■—). A second culture (—□—) was tested for growth by reinoculating SC + 2.0 M NaCl with surviving cells from the first culture (as obtained from colonies which grew on YEPD agar) after 30 hours of growth. The corresponding cell viability for the cell count at 8 days (192 hours) is approximately 50% (190 colonies/385 cfu plated).

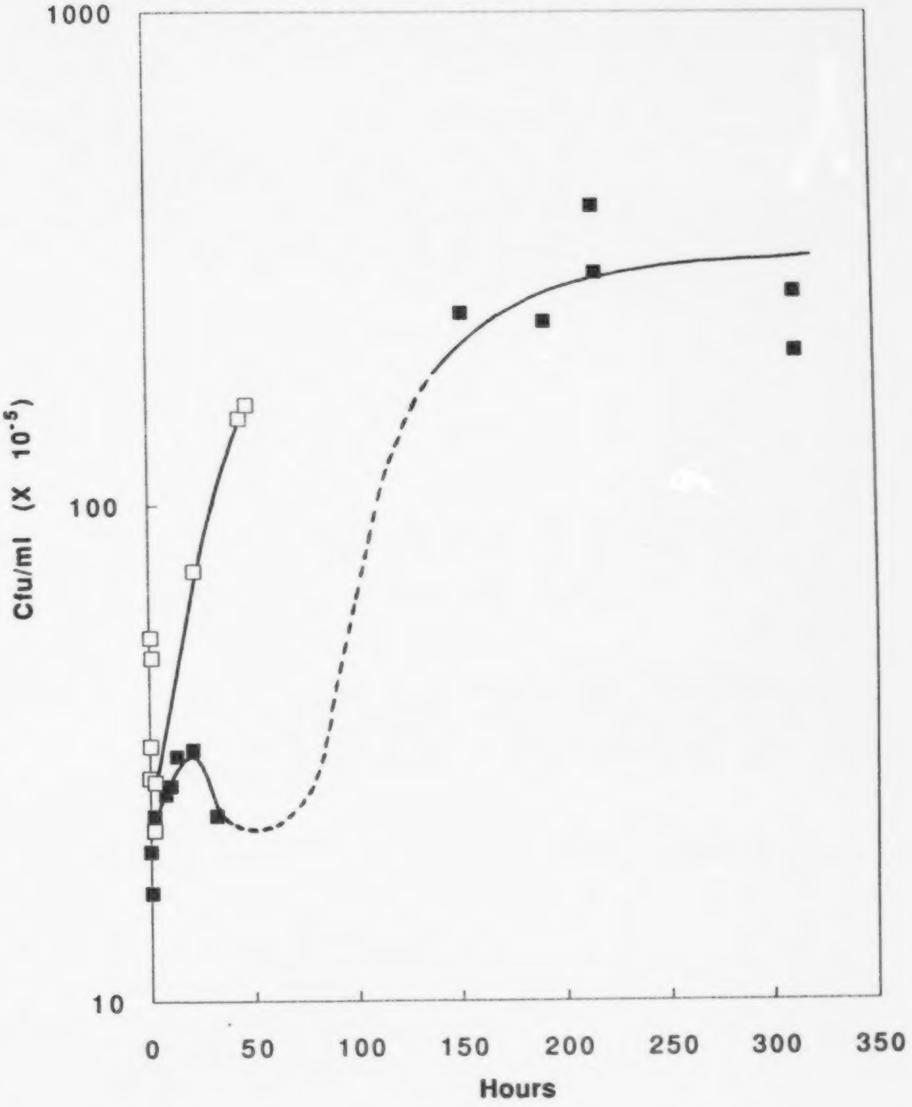

1000
100
10
Cfu/ml (X 10^{-5})
0
50
100
150
200
250
300
350
Hours

TABLE 2: Reversion frequencies in logarithmic phase cells of strain XV185-14C of *Saccharomyces cerevisiae* after treatment with 2 M NaCl or 2 M KCl

Treatment time (h)	Surviving fraction[a]	Reversion frequency[b] (revertants/10^7 surviving cells)		
		HIS$^+$	ARG$^+$	HOM$^+$
Control (water)				
0	1.0 (698)	9.6 (45)	1.7 (8)	1.9 (18)
Sodium chloride				
0.5	0.91 (637)	27 (117)	4.7 (20)	3.5 (30)
1.0	0.65	89 (270)	9.2 (28)	4.9 (30)
1.5	0.50	160 (380)	—	7.7 (36)
2.0	0.37 (261)	—	17 (29)	10 (36)
2.5	0.28	310 (406)	29 (38)	15 (39)
3.0	0.19	395 (352)	45 (40)	10 (18)
6.0	0.03 (21)	—	—	14 (4)
Control (water)				
0	1.0 (1084)	9.5 (81)	3.2 (27)	1.6 (27)
Potassium chloride				
1.0	0.67 (729)	69 (393)	6.6 (38)	4.3 (49)
1.5	0.42 (453)	140 (507)	18 (64)	8.2 (58)
2.5	0.23 (249)	320 (621)	35 (69)	16 (62)
3.0	0.16 (175)	350 (483)	39 (53)	18 (49)

[a] The figures in brackets represent the total number of colonies on 4 plates of YEPD-agar. Survival in the salt solutions is determined by ratio to control data. A dilution factor of 2×10^5 was used to obtain approximately 200 colonies per plate.

[b] The figures in brackets constitute the total number of revertant colonies on 4 plates of each omission medium (8 plates for SC-Thr). The number of cells per plate, as determined by hemocytometer, was 1.2×10^7 (for NaCl data) and 2.1×10^7 (for KCl data).

The data demonstrate that all three genetic markers revert when cells are treated with the salts, and that the mutagenic effect is similar quantitatively. For the 2.5-hour treatment time, the induction by NaCl of base substitutions at *arg4-17*, including its suppressors, and *his1-7* is 17-fold and 32-fold greater than the control levels, respectively; and KCl induces 11-fold and 34-fold increases for the same markers. Induction of frameshift mutations at *hom3-10* and its suppressors is increased by approximately 8- to 11-fold by NaCl and KCl.

Stationary phase cells exhibit only a slight decline in survival, and no significant change in reversion frequencies (Table 3). The apparent decline in survival at 2 and 6 hours in Table 3 is not significant because other experiments with the same molarity of NaCl and KCl reveal values closer to 95-100%. Figure 6, for example, shows that cell viability after 24 hours in 2 M KCl is 94% and this drops to 37% only after 5 days (data not shown). These survival data are similar to the survival of yeast cells resuspended in H_2O or 0.10 M phosphate buffer (see Figure 7 and Table 4), which suggests that the constraints upon viability after two days are probably determined more by starvation than osmotic effects. Figure 6 shows the effect of hypertonicity (2.0 M KCl) on cell survival over a treatment period of two days for cells that were removed at different phases of growth prior to treatment. As the cells approach higher cell concentrations, to a maximum of 6×10^8 cfu/ml in YEPD, a greater fraction of the population moves in to stationary phase and the populations clearly become progressively less sensitive to the lethal effects of the hypertonic conditions. Thus stationary phase cells, unlike logarithmic phase cells, are not susceptible to killing or mutagenesis by 2 molar salt solutions.

The lethal effects of osmotic shock are largely independent of the induction of base substitutions. When the severity of osmotic shock is reduced by exposing the cells to lower solute concentrations, the survival of the cells is increased (Figure 7) and the mutagenic response is delayed and/or decreased (Figures 8-10). In Figure 7, logarithmic phase cells that are resuspended in 0.60, 1.0 or

TABLE 3: Reversion frequencies in stationary phase cells of strain XV185-14C of *Saccharomyces cerevisiae* after treatment with 2 M NaCl

Treatment time (h)	Surviving fraction[a]	Reversion frequency[b] (revertants/10^7 surviving cells)		
		HIS$^+$	ARG$^+$	HOM$^+$
Control (water)				
0	1.0 (1021)	7.9 (47)	4.0 (24)	1.0 (12)
Sodium chloride				
2.0	0.95 (969)	9.0 (51)	2.6 (15)	0.88(10)
6.0	0.86 (881)	9.7 (50)	—	0.97(10)

[a] The figures in brackets represent the total number of colonies on 4 plates of YEPD-agar. Survival in the salt solutions is determined by ratio to control data. A dilution factor of 2 X 10^5 was used to obtain approximately 200 colonies per plate.

[b] The figures in brackets constitute the total number of revertant colonies on 4 plates of each omission medium (8 plates for SC−Thr). The number of cells per plate, as determined by hemocytometer, was 1.5 X 10^7.

TABLE 4: Reversion frequencies in strain XV185-14C of yeast after treatment with sodium saccharin

Treatment conditions		Surviving fraction[a]	Reversion frequency[b] per 10^7 surviving cells		
Solute, Molarity	Time (h)		HIS$^+$	ARG$^+$	HOM$^+$
Water (control)	0	1.00 (1050)	4.0 (38)	5.0 (47)	0.69 (13)
	20	0.96 (1014)	4.7 (43)	3.6 (33)	0.44 (8)
	40	0.84 (883)	6.2 (49)	3.4 (27)	0.38 (6)
	64	0.22 (236)	—	—	—
Sodium saccharin 0.01 M	20	0.94 (986)	5.3 (47)	4.4 (39)	0.57 (10)
	40	0.77 (813)	3.4 (25)	2.3 (17)	0.14 (2)
	64	0.23 (247)	—	—	—
0.10 M	40	0.62 (648)	6.4 (37)	3.8 (22)	0.52 (6)
	64	0.39 (412)	6.5 (24)	4.9 (18)	0.54 (4)
0.50 M	20	0.20 (213)	3.1 (6)	3.7 (7)	—
	64	0.11 (113)	6.9 (7)	4.9 (5)	0.99 (2)
Water (control)	0	1.00 (984)	7.0 (48)	3.6 (25)	4.1 (57)
Sodium saccharin 1.0 M	1	0.49 (482)	13 (43)	5.6 (19)	4.6 (31)
	2	0.27 (266)	12 (23)	3.8 (7)	10 (39)
	3	0.035 (136)	44 (21)	42 (10)	14 (13)
	4	0.0029 (58)	320 (13)	390 (8)	—

[a] The figures in brackets represent the total number of colonies on 4 plates of YEPD-agar. Survival in the saccharin solutions is determined by ratio to control data at time 0. A dilution factor of 2.0 X 10^5 was used to obtain approximately 275 colonies per plate, except for 3 and 4 hour treatments in 1.0 M sodium saccharin, where more cells were plated (dilution factors are 5.0 X 10^4 and 1.0 X 10^4 respectively).

[b] The figures in brackets constitute the total number of revertant colonies on 4 plates of each omission medium (8 plates for SC-Thr). The number of cells per plate, as determined by hemocytometer, was 1.2 X 10^7 (for NaCl data) and 2.1 X 10^7 (for KCl data).

FIGURE 6:

Effect of growth phase of a yeast cell population on survival after the cells are resuspended in 2 M KCl. The survival of yeast cells following osmotic shock (i.e., whereby the cells were resuspended in 2.0 M KCl after centrifugation to remove the growth medium) was determined from an overnight culture of strain XV185-14C in which cells were removed at the following stages of the growth curve prior to treatment: mid logarithmic phase, 6 X 10^7 cfu/ml (—▲—); late logarithmic phase, 1.35 X 10^8 cfu/ml (—□—); and stationary phase, 5.8 X 10^8 cfu/ml (—Δ—). Maximization of G_0-phase cells was obtained by resuspending a stationary phase population, at saturation of growth, in one volume of 0.1 M sodium phosphate buffer (pH 7.0) and incubating at 30°C overnight.

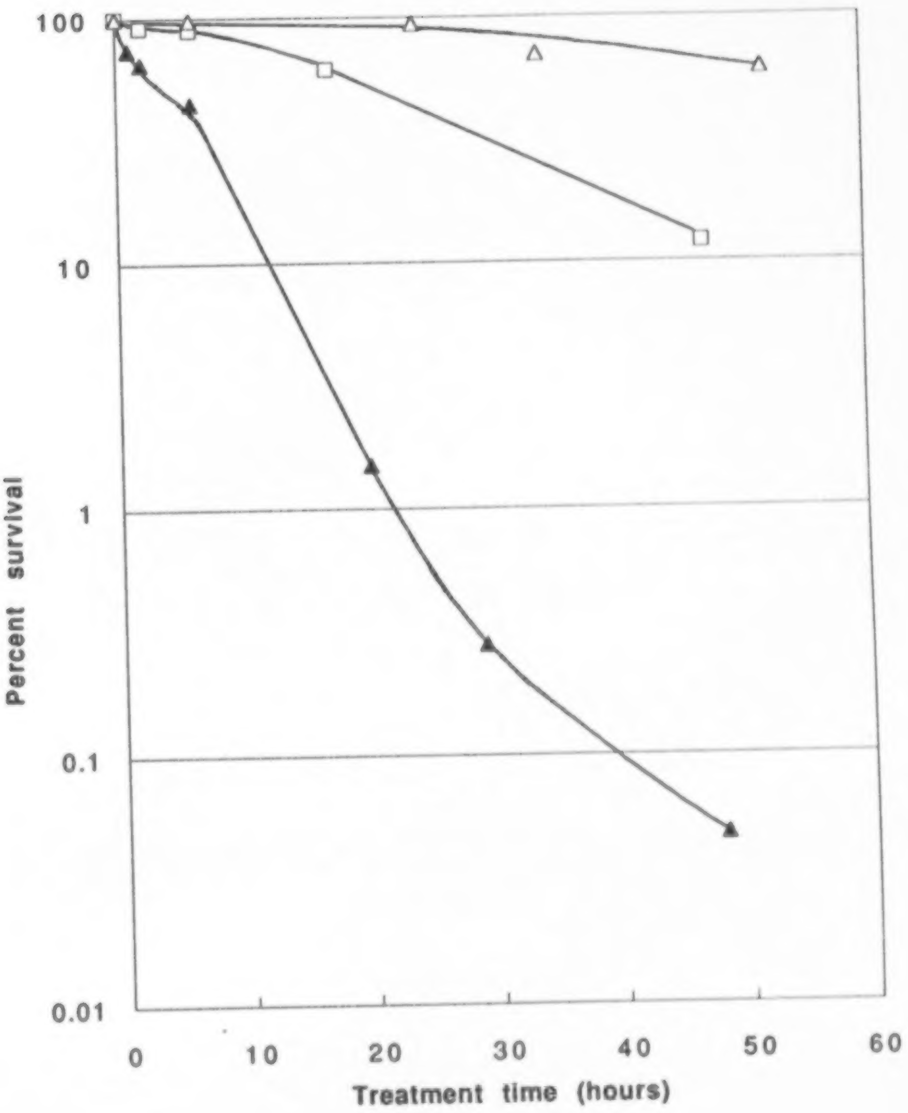
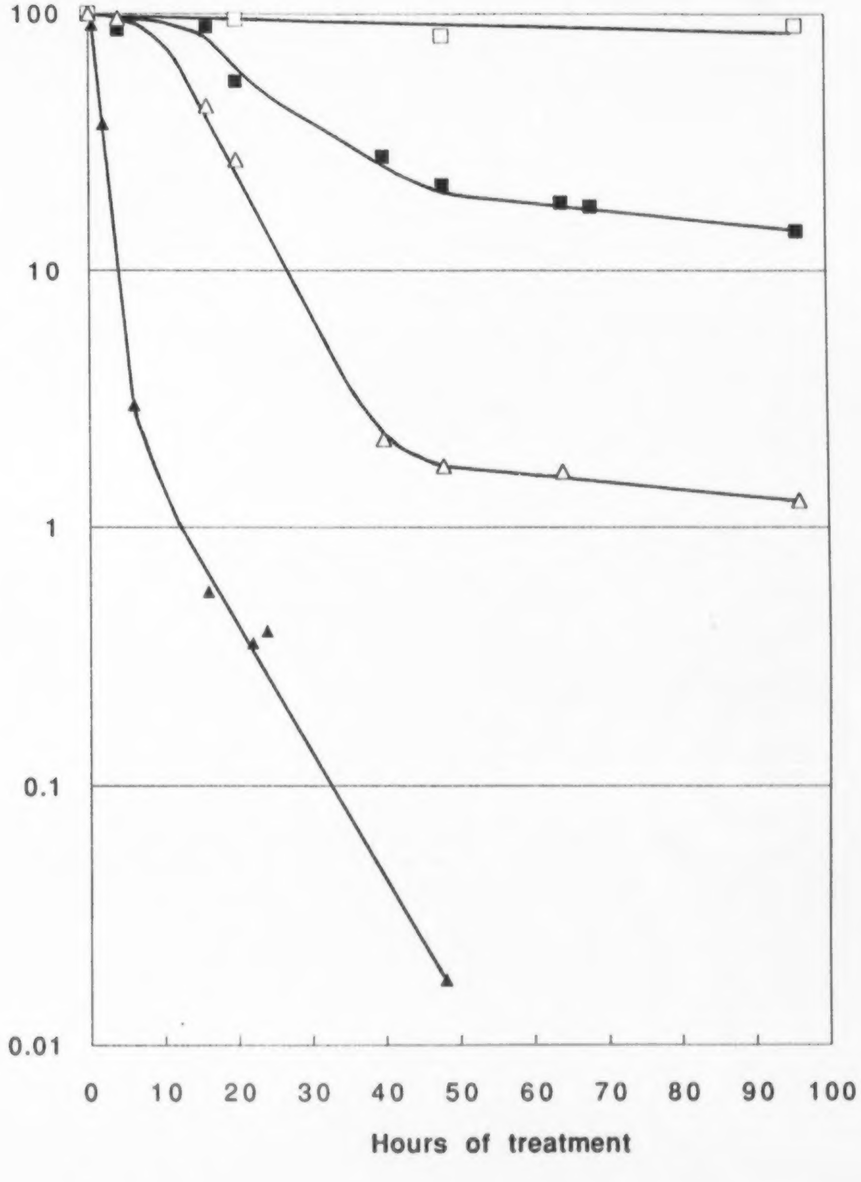

Percent survival
100
10
1
0.1
0.01
0
10
20
30
40
50
60
Treatment time (hours)

FIGURE 7:

Survival of logarithmic phase yeast cells after resuspension in different concentrations of NaCl. Logarithmic phase cells of yeast strain XV185-14C were harvested (at 5.65 X 10^7 cfu/ml) and resuspended in 0.10 M phosphate buffer (—□—), or 0.60 M (—■—), 1.0 M (—Δ—) or 2.0 M (—▲—) NaCl for up to 3 days. Surviving fractions (i.e., percent survival) represent the ratio of viable cfu (for different treatment times in NaCl) to that of the control at time 0, as determined by colony counts on YEPD plates.


100
10
1
0.1
0.01
0
10
20
30
40
50
60
70
80
90
100
Hours of treatment

2.0 molar NaCl (vs. 0.10 M phosphate buffer) exhibit a steeper decline in survival for the initial 40 hours (for cells treated in 0.60 M or 1.0 M NaCl) or 8 hours (for 2.0 M NaCl) of exposure, and thereafter the slope is decreased. This change in slope may be an indication that some cells in the population are less sensitive to the osmotic shock (e.g., 20% of the population is comparatively resistant to shock treatment in 0.60 M NaCl, but only 2% is similarly resistant to a 1.0 M shock); or it could represent the period of time required for removal of gene products actively involved in replication and mitosis, some of which might be damaging to the cell under the conditions of severe osmotic stress. The improved survival after 40 hours could be attributed to acquisition of stationary phase cells within the cell population, which are resistant to osmotic shock. However, the difference in the level of survival after 40 hours for cells treated in 0.60 M or 1.0 M NaCl cannot be due to a difference in the proportion of stationary phase cells at the outset of treatment because the cells for these two treatments derive from the same logarithmic culture. It thus seems likely that 40 hours represents a critical time period during which the cells induce a stress response which avoids the lethal effects of osmotic stress. Until such time as this response is fully induced (i.e., within the first 40 hours) the osmotic shock conditions exert a dose-dependent decrease in cell survival.

Resuspension of logarithmic phase cells in 1.0 M NaCl causes induction of reversions with a similar slope to that of 2.0 M NaCl for each of the three alleles tested in strain XV185-14C; however, the onset of the mutagenic response is delayed by 24 hours for *his1-7* (Figure 8) and *arg4-17* (Figure 9), and 8 hours for *hom3-10* (Figure 10). For reversion at *his1-7* and *hom3-10*, the slopes of the frequencies over time for 1.0 M NaCl are 1.4 and 1.5 which compares with 1.7 and 1.3, respectively for 2.0 M NaCl. In the case of *arg4-17*, two different experiments with 1.0 M NaCl (only one of which is presented in Figure 9) yield slopes of 2.3 and 2.9 which compares with 2.5 for 2.0 M NaCl. These results indicate that the slope measurements are reproducible for the same allele and that *arg4-17* is more sensitive to mutation.

FIGURE 8:

Reversion frequencies at *his1-7* induced by different concentrations of NaCl as a function of treatment time. Logarithmic phase cells of yeast strain XV185-14C were harvested (at 3.0-5.65 X 10^7 cfu/ml) and resuspended in 0.50 M (—■—), 0.75 M (—□—), 1.0 M (—▲—) or 2.0 M (—Δ—) NaCl for up to 3 days. Spontaneous reversion frequencies (···········) for different cell suspensions, as determined for control samples resuspended in H_2O or 0.10 M phosphate buffer (pH 7.0) at time 0 are 2.6 X 10^{-7} (for cell suspensions also treated in 0.50 M NaCl), 3.4 X 10^{-7} (for 0.75 M and 1.0 M NaCl), and 9.6 X 10^{-7} (for 2.0 M NaCl). The average spontaneous reversion frequency at intervals throughout the treatment period of 3 days is 4.2 ± 1.2 X 10^{-7} (for 0.75 M and 1.0 M NaCl).

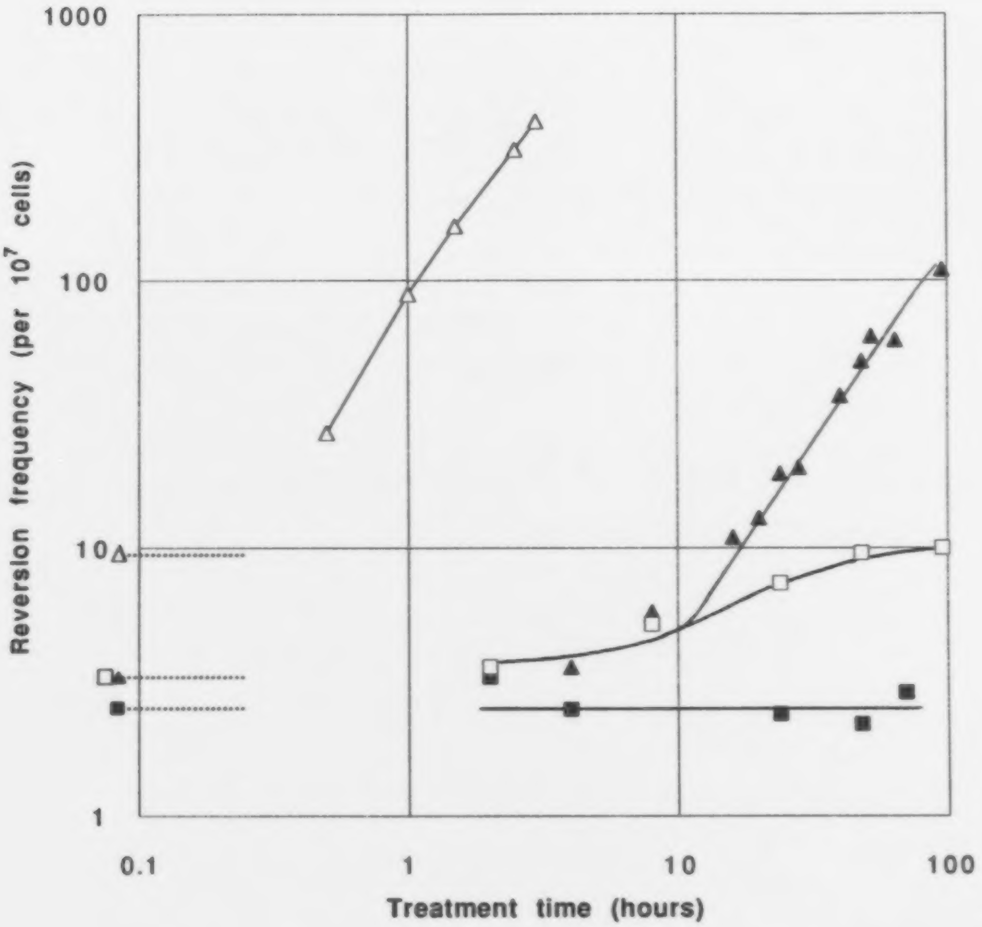

1000
100
10
1
0.1
1
10
100
Reversion frequency (per 10^7 cells)
Treatment time (hours)

FIGURE 9:

Reversion frequencies at *arg4-17* induced by different concentrations of NaCl as a function of treatment time. Cell suspensions as described in Figure 8 were also plated to SC-Arg to determine reversion frequencies at *arg4-17* (representing both reversions at the locus of *arg4-17* + mutations at external suppressor genes) after treatment in 0.50 M (—■—), 0.75 M (—□—), 1.0 M (—▲—) or 2.0 M (—Δ—) NaCl. Spontaneous reversion frequencies (············) in the absence of salt are 0.97 X 10^{-7} (for cell suspensions also treated in 0.50 M NaCl), 2.0 X 10^{-7} (for 0.75 M), 3.2 X 10^{-7} (for 1.0 M NaCl) and 1.7 X 10^{-7} (for 2.0 M NaCl) at time 0. Average spontaneous reversion frequencies as determined for control samples at intervals throughout the treatment period of 3 days are 3.2 ± 0.78 X 10^{-7} (for 0.75 M) and 3.2 ± 0.70 X 10^{-7} (for 1.0 M NaCl).

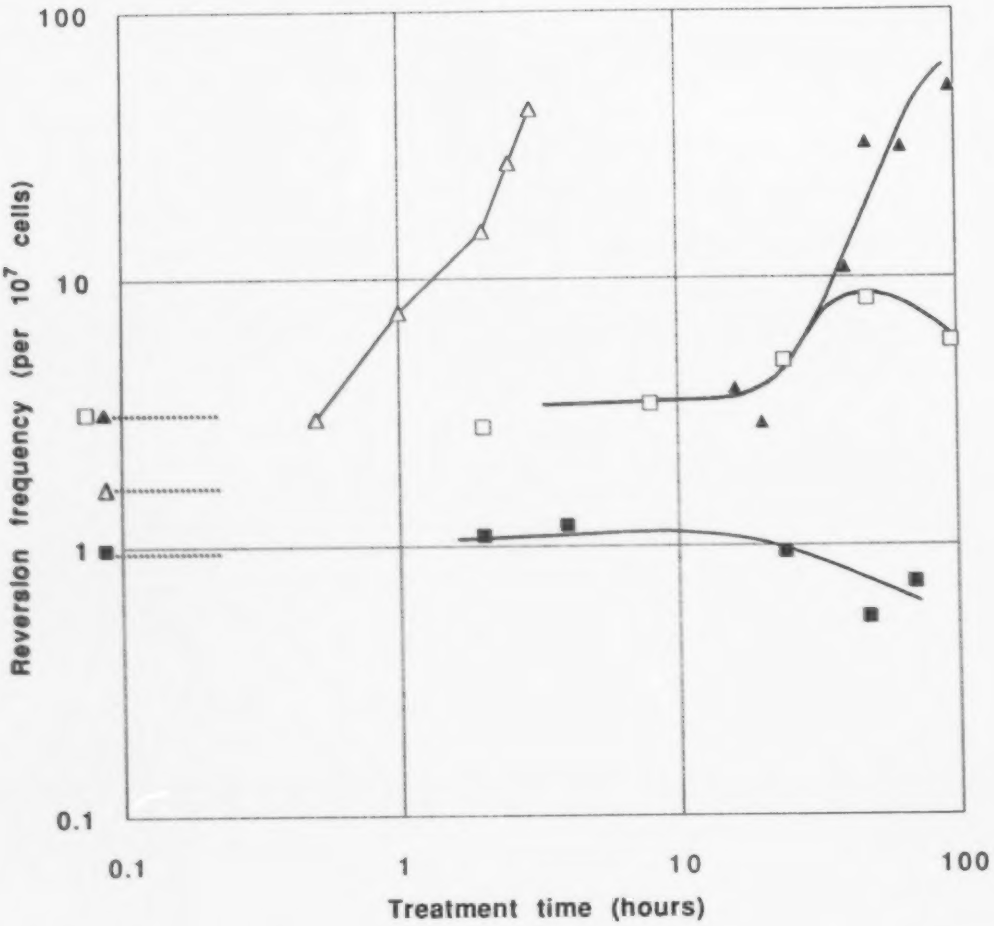
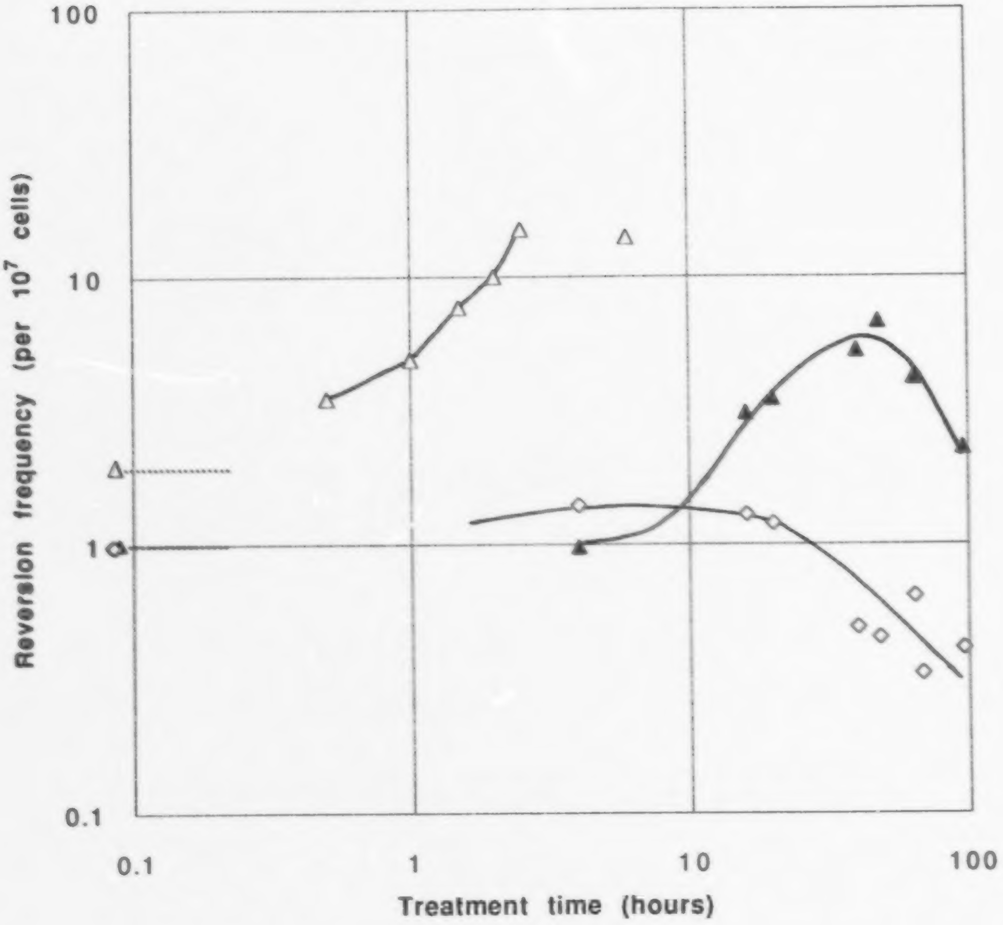

100
10
1
0.1
Reversion frequency (per 10^7 cells)
0.1
1
10
100
Treatment time (hours)

FIGURE 10:

Reversion frequencies at *hom3-10* induced by different concentrations of NaCl as a function of treatment time. Cell suspensions as described in Figure 8 were also plated to SC-Thr to determine reversion frequencies at *hom3-10* (representing both reversions at the locus of *hom3-10* and mutations at external suppressor genes) after treatment in 0.60 M (—◇—), 1.0 M (—▲—) or 2.0 M (—△—) NaCl. Spontaneous reversion frequencies (············) in the absence of salt are 0.98 X 10^{-7} (for cell suspensions also treated in 0.60 M and 1.0 M NaCl) and 1.9 X 10^{-7} (for 2.0 M NaCl) at time 0. The average spontaneous reversion frequency as determined for control samples (for 0.60 M and 1.0 M NaCl) taken at intervals throughout the treatment period of 3 days is 0.67 ± 0.23 X 10^{-7}.

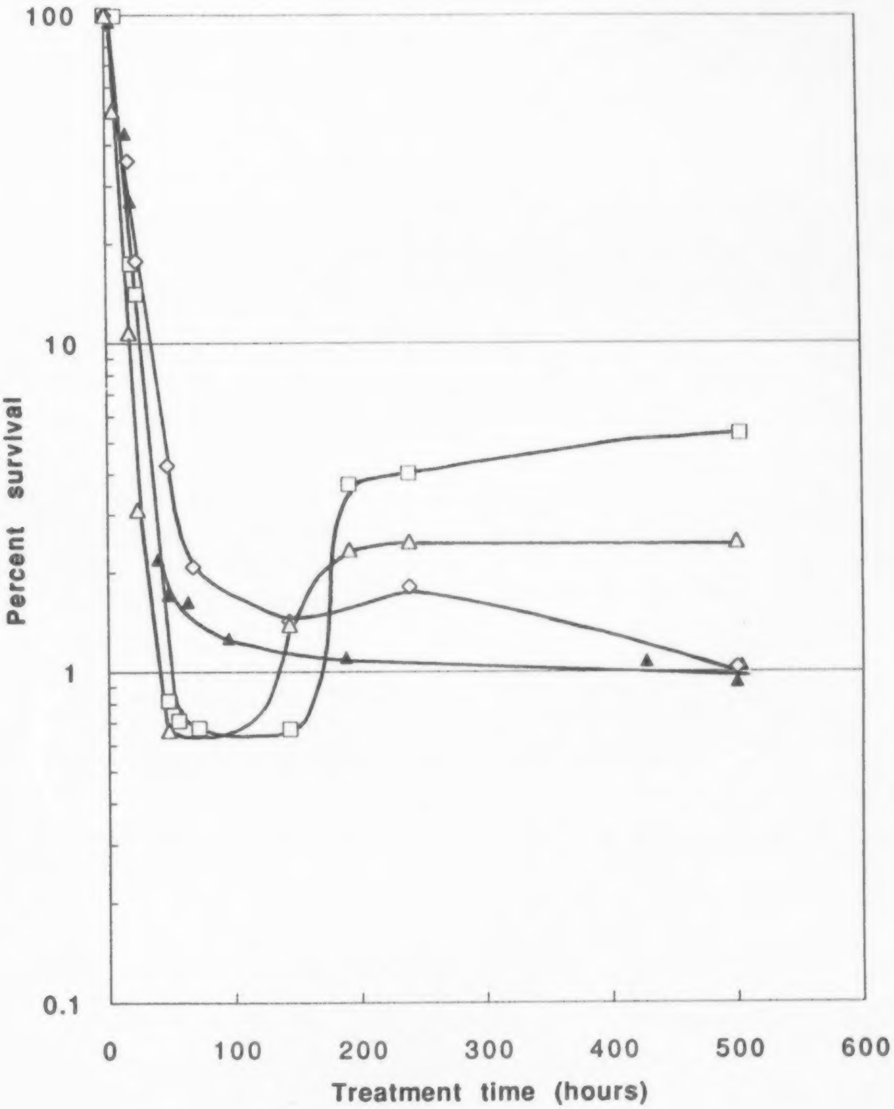

100
10
1
0.1
0.1
1
10
100
Reversion frequency (per 10^7 cells)
Treatment time (hours)

The increase in reversion frequencies for 1.0 M NaCl may be due to selection, given the prolonged treatment time of up to 3 days. This factor is ruled out by several lines of evidence: (1) The yield of revertants per total cells plated, Y(x), increases after 40 hours. This corresponds to the time when the slope of the killing curve is greatly reduced. After this adjustment, the decrease in survival between 40 and 96 hours is ~3-fold (Figure 7), but these surviving fractions allow for a 1.6-fold (21 → 35 revertants) and 2.8-fold (6 → 17 revertants) increase in the number of revertants per plate for *his1-7* and *arg4-17* (i.e., which correspond to the frequency values plotted in Figures 8 and 9), respectively. (2) The decrease in survival of logarithmic cultures of both revertant and non-revertant cells in 1.0 M NaCl, as shown in Figure 11, is in the range of two orders of magnitude, at least within a treatment period of three days. This survival plot also shows that HIS^+ and HOM^+ cells appear to be growing in the aqueous salt solution after 150 hours. The likely explanation for this growth is that a small amount of YEPD is carried over to the salt solution (i.e., since the cells were not washed following centrifugation). This permits a limited growth response among the surviving cells, though not until 4 days after osmotic shock is applied. (3) The spontaneous reversion frequencies for control samples resuspended in H_2O or phosphate buffer (as measured at time 0 and at intervals throughout the 3-day treatment period) decrease by ~2-fold for *hom3-10* and increase by ~2-fold for *his1-7* and *arg4-17* within 100 hours.

The reversion frequencies associated with exposure to 0.75 M NaCl are considerably reduced compared to 1.0 M NaCl, but the onset of mutagenesis occurs at about the same time (~24 hours) at these two concentrations. Resuspension of logarithmic phase cells in 0.50 or 0.60 M NaCl appears to bring about a 2-fold decline in the reversion frequencies for *hom3-10* after 24 hours (Figure 10), however the frequencies for control samples resuspended in H_2O or phosphate buffer show a similar decline during the same treatment period. Hence, this apparent antimutagenesis could not be attributed to osmotic stress. There is also an indication of antimutagenesis at *arg4-17* in which reversion rates decline by 1.8-fold after 24 hours for cells treated in

FIGURE 11:

Survival of revertants versus non-revertants in 1.0 M NaCl over a period of three weeks. Logarithmic phase cells of yeast strain XV185-14C (nonrevertants, —▲—) and of revertants of this strain to HIS1$^+$ (—□—), ARG$^+$ (—◇—) and HOM$^+$ (—Δ—) were harvested and resuspended in 1.0 M NaCl for up to 3 weeks. Surviving fractions (i.e., percent survival) represent the ratio of viable cfu (for different treatment times in NaCl) to that of the control at time 0, as determined by colony counts on YEPD plates. The cell cultures for the four strains with the above genotypes were harvested at 5.65 X 10^7, 2.5 X 10^7, 7.0 X 10^7, and 4.2 X 10^7 cfu/ml respectively.


100
10
1
0.1
Percent survival
0
100
200
300
400
500
600
Treatment time (hours)

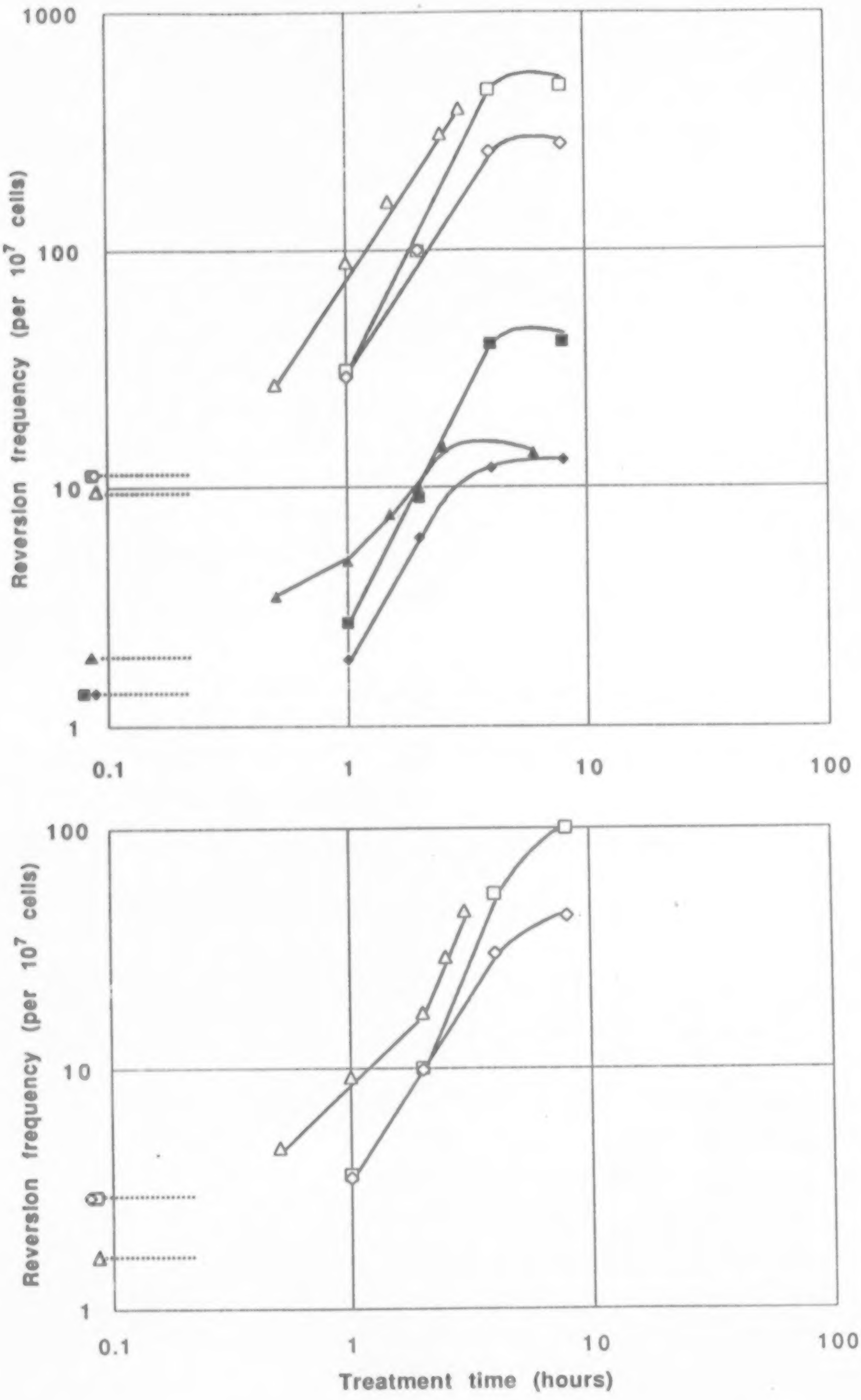
0.50 M NaCl (Figure 9). In the case of *arg4-17*, the reversion frequencies for control samples increase by ~2-fold throughout the 3 day treatment period. For *his1-7*, the antimutagenic effect is weaker (data not shown) and the control samples behave in the same way as *arg4-17* (Figure 8). Thus, when the severity of osmotic shock is lowered further from 1.0 M, the mutagenic effect in logarithmic phase cells is reduced within 20 to 40 hours so that reversion frequencies for base substitutions become slightly lower than control values.

In summary, the survival and reversion frequency data for yeast cells exposed to 0.50 to 1.0 M NaCl lead to the following conclusions: (1) The decrease in survival within 40 hours of treatment is largely due to events that are independent of mutation by base substitution. Evidence for this is clearly indicated by the absence of a change in reversion frequencies during the first 20 hours of exposure to 1.0 M NaCl or throughout the entire treatment period in the case of 0.50 or 0.60 M NaCl, when at the same time there is declining survival of the cells. Once the lethal effects diminish at 40 hours, any further decline in the surviving fraction (which could be associated with acquisition of lethal mutations) is in the range of 2-fold, rather than 2 orders of magnitude. (2) Reduction of the killing effect of osmotic shock at 40 hours does not alter the slopes of the reversion frequencies due to base substitutions. This verifies that revertants and nonrevertants are equally prone to the lethal effects. (3) The mutagenic effect of osmotic shock decreases when the concentration of NaCl is reduced from 1.0 to 0.50 or 0.60 M NaCl, such that the latter concentrations produce little or no effect on spontaneous mutation frequencies.

The mutagenic effects of osmotic shock are independent of solute chemistry, with some exceptions. It can be seen in Figure 12 that 1.0 M divalent salts ($MgCl_2 \cdot 6H_2O$ and $CaCl_2$) and 2 M monovalent salts (NaCl) yield approximately equivalent reversion frequencies for each of the three gene markers tested. This is apparent by the relatively constant slope (median = 1.7) for the logarithmic plots of reversion frequency against treatment time. An explanation for the similarity of the mutagenic response of these three salts is that the

FIGURE 12:

Reversion frequencies induced by monovalent and divalent salts as a function of treatment time. Logarithmic phase cells of yeast strain XV185-14C were harvested (at 3.15 - 4.4 X 10^7 cfu/ml) and resuspended in 1.0 M $MgCl_2 \cdot 6H_20$ (squares), 1.0 M $CaCl_2$ (diamonds) or 2.0 M NaCl (triangles) for up to 8 hours. The upper panel represents reversion frequencies for *his1-7* (open symbols) and *hom3-10* (closed symbols); the lower panel represents frequencies for *arg4-17*. Spontaneous reversion frequencies (············) in the absence of salt for *his1-7*, *hom3-10* and *arg4-17*.are respectively 9.6, 1.9 and 1.7 X 10^{-7} (for cell suspensions treated in NaCl) and 12, 1.4 and 3.0 X 10^{-7} (for cell suspensions treated in $MgCl_2$ and $CaCl_2$).


1000
100
10
1
0.1
1
10
100
Reversion frequency (per 10^7 cells)
100
10
1
0.1
1
10
100
Reversion frequency (per 10^7 cells)
Treatment time (hours)

solutions exert the same osmotic pressure, as determined by the numbers of solute and solvent particles. At these concentrations a similar response may be induced. The osmotic pressure is a colligative property of solutions which describes the hydrostatic pressure necessary to oppose the flow of water between pure water and a given solution separated by a semipermeable membrane (Hepler and Smith, 1975). Salts form osmotic pressures that are approximately proportional to the number of ions which dissociate in solution, although there are many other factors which influence this parameter such as the size of the solute, its electrolytic properties, and the interaction between solute and H_2O molecules. The osmolality of 2.0 M NaCl, at 3.84 Os/kg, is close to that of both 1.0 M $MgCl_2 \cdot 6H_2O$ and 1.0 M $CaCl_2$, at 3.46 (based upon extrapolation from published values) and 3.25 Os/kg, respectively. At these concentrations plasmolysis is clearly evident by microscopic examination of the cells, so that a loss of intracellular turgor pressure is not in question. Alternatively, these results could be an effect of the chloride ion since this anion is common to each of the three salts tested. Thus, if the mutagenicity of these different salts is a function of osmolality it will be necessary to show the generality of this theory with salts other than chlorides.

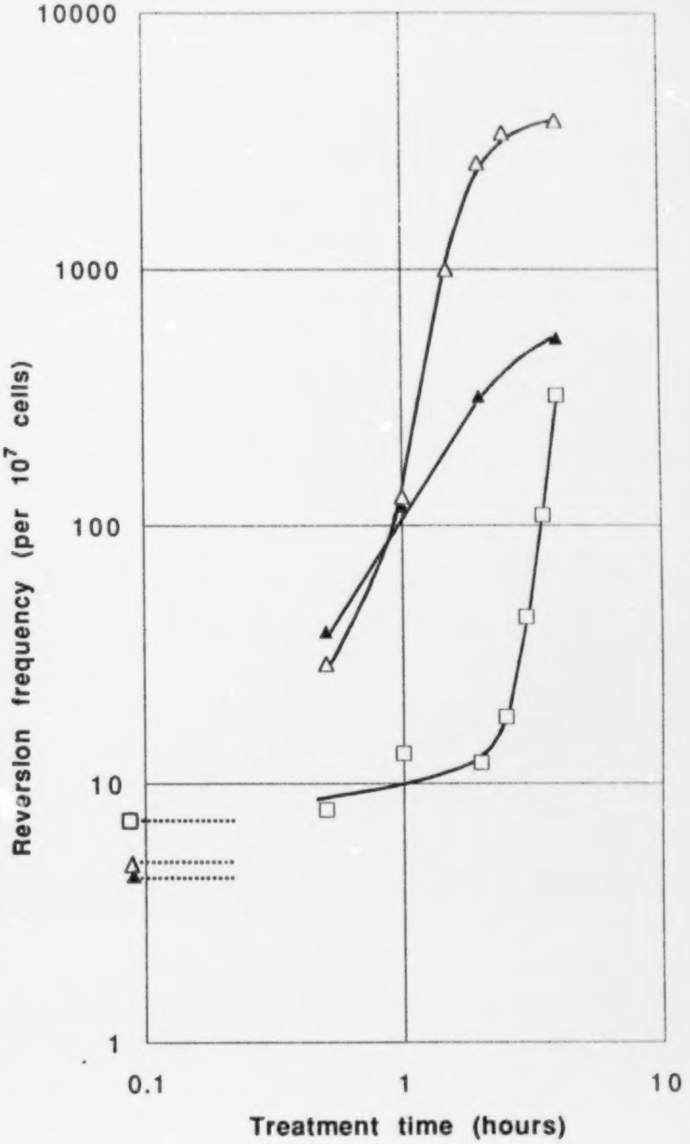
In the case of $MgCl_2$, the slopes in Figure 12 for reversion frequencies at *his1-7*, *arg4-17* and *hom3-10* over time are 2.0, 1.9 and 2.0, respectively. The corresponding slopes for $CaCl_2$ are 1.8, 1.6 and 1.7; and for NaCl, 1.7, 1.7 and 1.3. The value of 1.7 for reversion at *arg4-17* in 2.0 M NaCl is taken to be the average of two slopes of 0.96 and 2.5 (see Figure 12, lower panel). Other experiments with 2 M NaCl have produced slope measurements as follows, where unbracketed and bracketed values derive from experiments in which the dilutions of treated cells prior to plating were carried out in salt solutions isotonic with the treatment concentration (i.e. 2 M NaCl) and H_2O, respectively: 1.7 (2.4, 2.3), 1.7 (2.1, 2.3) and 1.5 (2.1). These data are quite consistent with the above values (obtained by dilution in isotonic saline solution), though it is apparent that dilutions in H_2O of cells previously suspended in 2 M NaCl heightens the mutagenic effect beyond a slope of 2. The slope of reversion frequency over time plotted on a logarithmic scale generally provides an indication of the kinetics of mutation, which in this case approximates 2 hits or partial hit kinetics (i.e. with a real value of 1.7). However, several factors may confound an interpretation of kinetic theory under these conditions, notably that the efficiency of DNA repair enzymes may be greatly influenced by osmotic shock. Thus, a slope of 1.7 could be a reflection of a reduced capacity to repair spontaneous lesions (including mismatches) rather than the kinetics of new lesions caused by osmotic shock.

The reversion frequencies at *his1-7* of two salts without chloride (Na_2S0_4 and sodium saccharin) and 2.0 M NH_4Cl are indicated in Figure 13. Both the sulphate and saccharin salts of sodium exhibit a mutagenic effect when cells are resuspended in 1.0 M solutions but the slope for sodium saccharin (6.1) is considerably higher than those in Figure 12. This is also the case for NH_4Cl which has a slope of 5.7. In further experiments with NH_4Cl as solute for osmotic shock and dilutions carried out with isotonic solutions of NH_4Cl rather than H_2O (data not shown), the mutations due to lower-order kinetics (with slopes ranging from 1.4 to 3.1 within the first 2 hours of treatment) and those involving higher-order kinetics (ranging from 4.0 to 9.8 after 2.5 hours) are set apart by a decline of the reversion frequency between 2.0 and 2.5 hours. These data indicate that mutations arising after a longer exposure to 2 M NH_4Cl are more complex, involving possibly as many as 10 events (e.g., slopes change from 2.3 to 9.8 for *arg4-17* after 2.5 hours), or that the ammonium ion (and the saccharin ion) exerts a more profound effect upon DNA repair enzymes. Since the osmolalities of NH_4Cl and NaCl are virtually identical (e.g., 3.89 vs. 3.84 Os/kg for 2 M NH_4Cl and 2 M NaCl, respectively), the steeper slopes of the reversion frequencies for NH_4Cl appear to refute the theory of mutagenesis by osmolality. One possible explanation for these deviations is that the ammonium and saccharin ions may gain entry to the cell more easily and thereby exert considerably stronger effects upon mutation.

When sodium saccharin is tested at 2 to 100 mg/ml (0.01 to 0.50 M) through a treatment period of 60 to 65 hours, which are the treatment conditions in which it was originally reported to be mutagenic and

FIGURE 13:

Reversion frequencies at *his1-7* induced by 2.0 M NH_4Cl, 1.0 M Na_2SO_4 and 1.0 M sodium saccharin. Logarithmic phase cells of yeast strain XV185-14C were harvested (at 2.6 X 10^7 cfu/ml) and resuspended in 2.0 M NH_4Cl (—Δ—), (at 3.6 X 10^7 cfu/ml) in 1.0 M Na_2SO_4 (—▲—) and (at 5.5 X 10^7 cfu/ml) in 1.0 M sodium saccharin (—□—). Spontaneous reversion frequencies (···········) in the absence of salt are 4.9 X 10^{-7} (for cell suspensions treated in NH_4Cl), 4.3 X 10^{-7} (for cell suspensions treated in Na_2SO_4) and 7.0 X 10^{-7} (for cell suspensions treated in sodium saccharin).


10000
1000
100
10
1
0.1
1
10
Reversion frequency (per 10^7 cells)
Treatment time (hours)

recombinagenic (Moore and Schmick, 1979a; 1979b) there is no indication of mutagenic activity in the haploid strain XV185-14C (Table 4). However, the conditions under which Moore and Schmick observed the mutagenic effects allowed for cell division of diploid yeast cells in a growth medium to which saccharin was added. This represents a very different test situation, as will be seen in part II of the results. These results indicate that conditions which induce mutagenesis by osmotic shock (i.e., resuspension of logarithmic phase cells in 1.0 M sodium saccharin) are not the same as those which lead to the expression of mutations in dividing cells, since the lower saccharin concentrations of 0.01-0.50 M fail to induce mutations in the absence of replication. Thus, osmotic shock appears to cause mutations through effects on intracellular water activity under conditions which induce cell cycle arrest and no further rounds of replication. On the other hand, osmotic stress may also induce mutations under conditions which allow for cell division (i.e., following an adaptive response to the increase in external osmolarity) providing that the collective osmolality of the medium plus that of added saccharin is high enough to constitute an osmotic stress stimulus. However, the finding that 0.01 M sodium saccharin (the lowest concentration tested) induces a 7-fold increase in reversion frequency and a 14-fold increase in the frequency of mitotic crossing over in a heteroallelic *ade2-119/ade2-40* diploid strain (Moore and Schmick, ibid) could be difficult to reconcile as a manifestation of osmotic stress, especially for a free-living microorganism such as *Saccharomyces cerevisiae*. On this basis, it is likely that saccharin is causing mutation and recombination that is unrelated to osmotic stress.

Several non-ionic compounds (glycerol, glucose and sorbitol) were tested for mutagenic effects at concentrations isoosmotic, or at least nearly so, with 2 M NaCl (2.85 M glycerol or glucose and 2.65 M sorbitol are isoosmotic with 2.0 M NaCl). As shown in Table 5, both 2.65 M glucose and 2.65 M sorbitol produce a similar mutagenic response to that of 2.0 M NaCl for each of the three genetic markers. At 1.8 M, sorbitol and glucose reduce the cell viability without, at the same time, inducing base substitutions (at least not within 18 hours of treatment). On the other hand, frameshift mutations are induced by both 1.8 and

TABLE 5: Effect of non-ionic solutes on logarithmic-phase yeast cells at concentrations iso-osmotic with 2 M NaCl

Treatment conditions		Surviving fraction	Reversion frequency[a] per 10^7 surviving cells		
Solute, Molarity	Time (h)		HIS$^+$	ARG$^+$	HOM$^+$
Water (control)	0.0	1.00	4.4 (44)	2.3 (23)	1.3 (13)
NaCl 2 M	2.5	0.12	350 (410)	26 (41)	11 (12)
Glycerol 2 M	3.5	1.00	5.1 (51)	2.1 (21)	0.96 (10)
Glucose 1.8 M	6.0	0.57	7.2 (41)	2.5 (14)	3.7 (21)
	18	0.44	5.4 (24)	2.6 (12)	3.2 (14)
2.65	2.0	0.26	390 (1000)	21 (55)	13 (34)
Sorbitol 1.8 M	6.0	0.43	11 (48)	4.6 (20)	6.1 (27)
	18	0.23	5.8 (13)	2.3 (5)	5.3 (12)
2.65	2.0	0.24	300 (720)	30 (72)	16 (38)

[a] Reversion frequencies are calculated per 10^7 surviving cells based on the surviving fractions of cells on YEPD after treatment in the hypertonic solutions. Revertant yields (indicated in brackets) standardized per 1.0 X 10^8 plated cells correspond approximately to the number of revertant colonies scored on 4 test plates.

2.65 M glucose or sorbitol, but not 2.0 M glycerol, within at least 6 hours of treatment. These results resemble those described for NaCl, where it is seen that the lethal effects of osmotic shock are largely independent of base substitution and, secondly, that frameshift mutations are expressed earlier and separate from base substitutions. These data add further support to the hypothesis that osmotic shock, as determined by the permeability of solutes across the cellular membrane but not their chemical properties, can give rise to both lethal and mutagenic effects in logarithmic phase cells. The basis for these two effects could be a function of direct effects of the loss of intracellular water activity and/or a disruption of ionic homeostasis upon the activity of enzymes engaged in replication and mitosis.

Components in the growth medium can prevent the induction of base substitutions by osmotic shock. The previous experiments have shown that osmotic shock involving replacement of the growth medium by aqueous hypertonic solution causes point mutations of all types and considerable loss of viability in logarithmic phase cells. Since all microorganisms utilize certain low molecular weight components to protect the cells against the damaging effects of osmotic shock, the removal of the growth medium may deplete the supply of these compounds. Table 6 presents the results of experiments in which osmotic shock was induced by adding the hypertonic solutions to the growth medium so that the potential effect of these compounds might be seen. Tests were conducted with two haploid yeast strains, ES55-6A and ES63-11A, both of which harbour an integrating plasmid with a wild type copy of the *URA3* gene. These strains were constructed to assess forward mutations at the *URA3* gene, as well as reversions at ochre-suppressible alleles. The DNA from *ura3*$^-$ mutants could then be extracted, transformed in to *Escherichia coli* to select and amplify the plasmid, and sequenced. ES55-6A carries the *ura3-52* gene in which the coding frame of *URA3* is disrupted by a Ty element insertion. In ES63-11A the genomic copy of *URA3* is deleted.

Addition of high concentrations of saline solution to a growing yeast cell population causes immediate cessation of cell division, as

TABLE 6: Amelioration of osmotically-induced mutations in strains ES55-6A and ES63-11A by components in the growth medium

Treatment conditions[a]	Time (h)	Medium	Percent survival	Yield[b] (per 10^8 plated cells)			Frequency[c] (per 10^7 surviving cells)		
				ARG4$^+$	*SUP*$^+$	*ura3*$^-$	*ARG4*$^+$	*SUP*$^+$	*ura3*$^-$
ES55-6A									
H_2O	0.0	none	100	5.3	19	< 86	0.53	1.9	< 8.6
	4.0		120			71		7.1	
NaCl	1.0		46	3.2	18	1900	0.70	3.9	28
	2.0		3.4			4400			1300
	3.0		2.0	9.6	29	2300	47	140	1100
	4.0		0.86	3.2	11	1300	38	130	1500
H_2O	0.0	SC	100	15	6.2	120	1.5	0.62	12
NaCl	2.0		60	9.7	19	680	1.6	3.3	110
H_2O	0.0	YD	100	10	12	550	1.0	1.2	55
NaCl	2.0		58	11	5	540	1.8	0.91	94
H_2O	0.0	YD	100	10	6	170	1.0	0.62	17
NaCl	18[a]		100	11	9		1.1	0.90	
	1.0		110	14	6	130	1.4	0.60	13
	2.0		90	14	9	210	1.6	1.1	23

ES63-11A

H_2O	0.0	SC	100	< 0.48	78	59	< 0.048	7.8	5.9
NaCl	2.5		8.3	3.2	64	1400	3.8	77	1700
H_2O	0.0	SC	100	< 0.78	18	< 7.8	< 0.078	1.8	< 0.78
NaCl	2.5		16	4.1	20	360	2.6	13	230
H_2O	0.0	YD	100		22	170		2.2	17
NaCl	2.0		43		8.9	640		2.1	150
	6.0		12		3.5	850		2.9	700
H_2O	0.0	YD	100		51	220		5.1	22
NaCl	2.0		56		29	1300		5.1	230
	6.0		22		970			440	

---

[a] Logarithmic phase cells were treated in 2.0 M NaCl, except for one 18 hour treatment in 1.0 M NaCl.

[b] Yield represents the number of *ARG*$^+$ revertants (either *ARG4*$^+$, by reversion at the ochre site, or *SUP*$^+$, by external suppressor mutation) and *ura3*$^-$ mutants per 10^8 plated cells.

[c] Frequency represents the number of mutational events per 10^7 surviving cells.

indicated by cell counts taken before and after the osmotic shock. Figure 5 shows that the cells can eventually undergo division in a medium such as SC at 2 M NaCl, but not until after a lag time of more than 30 hours. With these considerations in mind, tests of osmotic shock were usually conducted within a 1 to 6 hour treatment period before such time as the surviving cells can resume division. One experiment with strain ES55-6A allowed for an 18 hour exposure to 1.0 M NaCl in YEPD (Table 6), during which the cells underwent one cell generation.

Yeast strain ES55-6A, when subjected to osmotic shock after removal of the growth medium, shows an increase of close to 90 and 75-fold for reversion frequencies at *arg4-17* (locus revertants and suppressors, respectively) and ~200-fold for forward mutations in the *URA3* gene within 3 hours of treatment (Table 6). The effects at *arg4-17* seen here for cells harvested at 8.5×10^6 cfu/ml are somewhat greater than those reported in Table 2 (25-fold at 3 hours) for strain XV185-14C harvested at 4.4×10^7 cfu/ml. This is so because the viability of strain ES55-6A under these conditions is substantially less than that of XV185-14C. Consequently, the reversion yields in Table 6 are not more than 2-fold higher than the spontaneous values for both locus revertants ($5.3 \rightarrow 9.6$ per 10^8 cells) and suppressors ($19 \rightarrow 29$ per 10^8 cells).

When the osmotic shock is applied by adding one volume of 4 M NaCl to a logarithmic yeast cell population in SC or YEPD medium, thereby increasing the molarity of the medium to 2 molar, the lethal and mutagenic effects are markedly reduced compared to the treatments in which the growth medium is first removed and the cells are then resuspended in an aqueous 2 M salt solution. For a salinized SC medium, the reversion frequencies for suppression at *arg4-17* after a 2 to 2.5 hour treatment are 5- to 10-fold greater than spontaneous levels, as shown for both of the haploid strains in Table 6. By comparison, a salinized YEPD medium produces no increase in the reversion of alleles requiring a base substitution (i.e., *ARG4*$^+$ and *SUP*$^+$), at least not within the first 2 hours after salinization. Strain ES55-6A exhibits very little effect of osmotic shock on locus reversion at *arg4-17*, and forward mutation frequencies at *URA3* drop to a 9-fold increase (12 → 110 per 10^7 surviving cells) for SC + 2 M NaCl and below a 2-fold effect (55 → 94 per 10^7 surviving cells) for YEPD + 2 M NaCl. In contrast, the presence of SC medium does not reduce the mutagenic effects of osmotic shock in strain ES63-11A, since reversion frequencies for base substitutions to ARG^+ are still increased by 30 to 80-fold and forward mutation frequencies to *ura3*$^-$ are increased by ~300-fold. The presence of YEPD appears to delay the mutagenic response, as indicated by the induction of suppressor mutations at *arg4-17* after 6 hours, and causes a substantial decrease in the forward mutation frequencies to *ura3*$^-$ so that a 2 hour treatment produces only a 10-fold increase. Reversion at *trp5-48*, which is suppressible by a broader range of base substitutions than *arg4-17*, shows a 3-fold increase in frequency but no increase in yield after two hours of treatment in YEPD + 2 M NaCl (data not shown). The increase is 8-fold in SC + 2 M NaCl. Hence, these results suggest that YEPD medium, and to some extent SC medium, can alleviate or at least delay most of the lethal and mutagenic effects of osmotic shock. The growth medium may pre-condition the cells in some way to prevent the induction of mutations or it may contain low molecular weight osmolytes which can act as osmoprotectants.

Ura3$^-$ mutations induced by osmotic shock in the presence of growth medium (SC or YEPD) were sequenced for strain ES63-11A. These data are presented in Tables 7 and 8. For comparison, the spontaneous mutations which occur in the absence of NaCl for strain ES63-11A and two other strains are shown in Tables 18 (mutation spectra), 23 (observed base substitutions) and 25 (observed frameshift mutations).

The mutations are grouped according to mutation type in the spectra presented in Tables 7 and 18. Only three base substitutions were detected among the 19 mutations sequenced, none of which affected A•T sites. Although frameshift mutations comprise an unusually large fraction (9/26 = 35%) of the total spontaneous mutations for strain ES63-11A (cf. strains XV185-14C and QW6-2A) in the absence of NaCl (Table 18), the proportion (10/11 = 91%) is significantly increased by osmotic shock for cells growing in YEPD (Table 7). Even with the limited number of mutations sequenced, it is readily apparent from

TABLE 7: Mutation spectra for *ura3* mutations obtained from yeast strain ES63-11A after osmotic shock in YD or SC + 2.0 M NaCl

Mutation type	Osmotic shock conditions	
	YD + 2.0 M NaCl	SC + 2.0 M NaCl
Base-pair substitutions:		
G•C → A•T		1
G•C → T•A		1
G•C → C•G	1	
Deletions:		
– G or C	7	3
– A or T	2	2
Replacements:		
TGC → G	1	
CG → A		1
TOTAL	11	8

these spectra that osmotic shock is mostly inducing frameshift mutations, particularly those involving deletion of G•C base pairs. This type of mutation seldom occurs in the spontaneous spectra of any of the three strains represented in Table 18 (e.g., 2 per combined total of 89 mutations). A majority (6/8 = 75%) of frameshift mutations is also observed in the mutation spectrum of cells obtained from SC with osmotic shock (Table 7). The occurrence of two different base substitutions in this spectrum can be attributed to the osmotic shock rather than incidental isolation of spontaneous *ura3*$^-$ mutations among the cell population prior to treatment, since the forward mutation frequencies following osmotic shock are ~300-fold greater than the spontaneous frequencies without treatment (Table 6). Thus, these data concur with the reversion data which show that base substitutions become less frequent as the nature of the osmotic shock is changed from one that has virtually no osmolytes to one in which these components could at least be a factor.

The base substitutions and frameshift mutations induced in the *URA3* gene by osmotic shock are shown in Table 8. The mutations are equally divided between the N-terminal (10/19 = 53%) and C-terminal (9/19 = 47%) halves of the gene. More than one-third (7/19) of the mutations, including 2 of 3 base substitutions, all of the -1 deletions affecting A•T residues, and one -1 deletion of a G•C base pair have been encountered in the spectrum of spontaneous mutations without NaCl (cf. Appendices 3 and 5). The C•G → A•T transversion at position 461 has been seen in the mutation spectrum of cells undergoing division in NaCl-supplemented medium (Appendix 4), which is an aspect of osmotic stress to be presented in part II. However, most of the mutations (11/19 = 58%) occur at unique sites within the gene, as a reflection of the tendency of osmotic shock to induce specifically frameshift mutations affecting G•C residues. These sites often show iteration of G or C nucleotides, but there are also 4 noniterated positions which give rise to single base deletions and some peculiar replacements.

TABLE 8: Base pair substitutions and frameshift mutations at the *URA3* gene induced by osmotic shock in 2.0 M NaCl in yeast strain ES63-11A

Base position[a]		Mutation	Sequence context (nontranscribed strand)
14		– C•G	GAAAGCTACATATAA$_{20}$
89		C•G → G•C	AAAGCAAACAAACTT$_{95}$
174-178		– A•T	ACTAAAAACACATGT$_{185}$
201-205	**	– T•A	TCTTGACTGATTTTT$_{205}$
206-207		– C•G	CCATGGAGGGCACAG$_{220}$
255-260		– T•A	CAAGTACAATTTTTT$_{260}$
273		– C•G	GACAGAAAATTTGCT$_{285}$
344-346		– G•C	AATGGGCAGACATTA$_{355}$
378-380		– C•G	TGGTGGGCCCAGGTA$_{385}$
437		G•C → A•T	CTAGAGGCCTTTTGA$_{445}$
461		C•G → A•T	GCAGAATTGTCATGC$_{465}$
498-500		– G•C	ATACTAAGGGTACTG$_{505}$
540-541		CG → A	ATCGGCTTT$_{546}$ → ATAGCTTT
607-608		– G•C	ACACCCGGTGTGGGT$_{615}$
700-701		– G•C	ATTATTGTTGGAAGA$_{705}$
720-722		– G•C	CAAAGGGAAGGGATG$_{730}$
725-727		– G•C	
729, 731		–T, –C	CTAAGGTAGAGGGTG$_{745}$
TOTAL	19		

[a] Base positions correspond to the sequence of nucleotides in the open reading frame of the *URA3* gene, delimited by ATG$_3$ and TAA$_{804}$ in the transcribing strand. Duplicate mutations are designated by *. The left nucleotide of each base pair corresponds to the nontranscribed strand of DNA.

B. Interchromosomal recombination in diploid cells

The effects of osmotic shock on mitotic recombination were assessed in the diploid strain, D7-144. Survival data and recombination frequencies for logarithmic phase cells treated with 2 M NaCl are presented in Tables 9 and 10. In Table 9, the frequencies per 10^3 cells describe 2 types of recombinational events which lead to homozygosis of the heteroallelic markers, *ade2-40* and *ade2-119*, carried in this strain. Mitotic crossing over is based on the number of sectored pink/red and pink/red/white colonies, and mitotic conversion on the number of coloured (pink or red) and white-sectored (pink/white or red/white) colonies. The tabulated numbers of colonies summarize the results from 3 separate experiments in which the average cell concentration of the cultures prior to treatment was 1.4 X 10^7 cfu/ml. A cell culture at 2.8 X 10^8 cfu/ml gives recombination frequencies similar to those of cultures taken at lower cell titres; however, a rigorous test of the capacity of osmotic shock to increase mitotic recombination in a stationary phase culture, was not conducted.

Osmotic shock increases the frequencies of mitotic crossing over and conversion at the *ade2* gene (Table 9) and intragenic recombination at the *trp5* gene (Table 10). Relative to the controls (i.e., cells resuspended in water), conversion frequencies are increased by 20 to 30-fold for cells resuspended in 2 M NaCl and then diluted in either H_2O or isotonic NaCl after a 2 hour treatment in 2 M NaCl. Conversion is more frequent at *ade2* by ~2 orders of magnitude than at *trp5*, because the latter events are confined to those conversion tracts which terminate between *trp5-12* and *trp5-27* to generate a *TRP5*$^+$ allele. The frequency of mitotic crossing over is increased by at least 30 to 80-fold compared to the frequencies of the corresponding control cells; however, the frequencies for the control groups are based on the absence of a single crossover event among 6,000 or 9,000 plated cells. A summary of all the spontaneous recombinant events at the *ade2* gene for untreated cells (top line in Table 9) reveals only one crossover in 36,000 cells. Therefore, the frequency of mitotic crossover following osmotic shock is more in the range of 100 to 500-fold higher than the spontaneous frequency of 2.8 X 10^{-5}.

The basis for the increase in recombination frequencies can be seen in Figures 14 to 16, where the recombinant yields and surviving fractions are plotted separately. The values for these graphs are taken from Tables 9 and 10. Osmotic shock increases the yields of conversion at *ade2* by 3 to 4-fold (Figure 15) and at *trp5* by 6-fold (Figure 16), but it increases the yield of mitotic crossing over by ~60-fold (Figure 14). These results show that osmotic shock increases the yield of recombinational events apart from its effects on cell survival, and that a much larger fraction of the interchromosomal exchanges result in crossing over.

The survival plots in Figures 14 and 15 indicate that the decline in survival of recombinants (i.e., *ade2-x/ade2-x*) is parallel to that of the *ADE2*$^+$ (i.e., *ade2-40/ade2-119*) population. This means that the killing effects apply equally to both types of cells, which in turn strengthens the validity of the frequency measurements in Table 9. On the other hand, the decline in survival of *TRP5*$^+$ recombinants (i.e., *TRP5*$^+$*/trp5-x*) is less than that of the *trp5*$^-$ (i.e., *trp5-12/trp5-27*) population (Figure 16), presumably due to selection. This gives reason to doubt the conversion frequencies in Table 10, although it would appear that only the frequency at 4 hours (i.e., 120) is clearly affected.

Osmotic shock leads to higher lethality in the diploid strain D7-144 than any of the haploid strains tested under the same conditions. Surviving fractions for strain D7-144 are 0.39 (Table 9) or 0.43 (Table 10) after 1 hour of treatment in 2 M NaCl and 0.046 or 0.081 after 2 hours, when the samples are diluted in isotonic (2.0 M) NaCl following treatment. By comparison, the haploid strain XV185-14C shows survival values of 0.65 for 1 hour and 0.37 for 2 hours (Table 2). For samples diluted in H_2O following treatment in 2 M NaCl, the survival is reduced even further. Comparable values for a haploid strain tested under these dilution conditions are provided in Table 6 for strain ES55-6A (without medium). These data also show better survival for the haploid strain, which seems contrary to the expectation that the diploid strain should have improved survival because of the availability of a homolog for repair of chromosome breaks.

Further experiments using other diploid strains (including D7, which is sporulation proficient) indicates that the extreme sensitivity to osmotic shock of strain D7-144 is a peculiarity of this strain (data not shown). Thus, diploid strains are not more sensitive than haploids, but it is not apparent that diploids are more resistant either. Further experiments are needed to clarify this point.

In view of the unusual sensitivity of strain D7-144 to osmotic shock, tests were conducted with strain D7 to ascertain whether the elevated levels of recombination were not also associated with the sporulation deficiency of strain D7-144. The results show that osmotic shock causes an increase in interchromosomal recombination, particularly mitotic crossing over (data not shown).

TABLE 9: Mitotic recombination at the *ade2* gene of strain D7-144 of *Saccharomyces cerevisiae* after treatment with 2 M NaCl

Dilution conditions[a]	Time (h)	Percent survival	Number of cells plated (X 10^{-3})	Number of *ade2* colony types[b]						Recombination frequency (X 10^{3})	
				p	r	p/w	r/w	p/r	p/r/w	crossover	conversion
Control[c]	0	100	36	8	12	0	2	1	0	0.028	0.61
H_20	0	100	5.8	3	0	0	0	0	0	< 0.17	0.51
	0.5	57	4.5	1	1	2	2	3	0	1.2	2.3
	1.0	24	32	34	15	5	5	20	1	2.8	7.7
	2.0	0.99	26	1	2	0	1	4	0	16	16
NaCl	0	100	9.3	6	7	0	1	0	0	< 0.11	1.5
	0.5	76	7.0	6	4	13	6	10	1	2.2	5.9
	1.0	46	21	52	28	11	18	20	2	2.7	14
	2.0	4.6	13	11	7	0	0	2	0	3.3	30
	4.0	0.041	1200	6	7	0	2	3	0	6.9	36

[a] Dilutions following treatment in 2.0 M NaCl for 1 to 4 hours were in either water or 2 M NaCl (i.e, isotonic to the treatment concentration).

[b] Colonies of six types represent the totals obtained on YEPD plates from 4 separate experiments, where r = red (*ade2-40/ade2-40*), p = pink (*ade2-119/ade2-119*) and w = white (*ade2-40/ade2-119*) phenotypes (genotypes). Sectored colonies due to crossing over are indicated by p/r and p/r/w; all other colonies are grouped as conversion events.

[c] "Control" (top line) is a summary of results without NaCl exposure, including an additional test to approximate the spontaneous frequency of mitotic crossing over.

TABLE 10: Mitotic recombination at the *trp5* gene of strain D7-144 of *Saccharomyces cerevisiae* after treatment with 2 M NaCl

Dilution conditions[a]	Time(h)	Percent survival	Number of cells plated (X 10^{-7})	Number of TRP$^+$ colonies	Conversion frequency[b] (X 10^5)
H_2O	0	100	12	829	0.69
	0.5	68	2.4	789	4.9
	1.0	26	1.8	731	16
NaCl	0	100	24	1472	0.62
	0.5	72	3.6	1231	4.7
	1.0	43	2.8	1019	8.6
	2.0	8.1	12	804	8.3
	4.0	0.029	24	81	120

[a] Logarithmic phase cells of diploid strain D7-144 were resuspended in 2 M NaCl after one wash to remove traces of YEPD medium. Dilutions following treatment in NaCl for 1 to 4 hours were in either water or 2 M NaCl.

[b] Conversion frequencies are calculated from the number of TRP$^+$ prototrophs per 10^5 surviving cells on SC-Trp plates, based on the surviving fractions of cells on YEPD after treatment in the hypertonic solutions.

FIGURE 14:

Surviving fraction and yield of mitotic crossovers in strain D7-144 after treatment with 2 M NaCl. Logarithmic phase cells of yeast strain D7-144 were harvested (at 1.4 X 10^7 cfu/ml, the average from 4 separate experiments) and resuspended in 2.0 M NaCl for up to 4.0 hours. An aliquot of this cell culture was also resuspended in H_2O as a control. Percent survival, S(x), represents the ratio of viable cfu at time x to that of the control at time 0, as determined by colony counts on YEPD plates. Samples treated in NaCl were diluted in either H_2O (—▲—) or 2 M NaCl (—Δ—) prior to plating. The yield of mitotic crossover, Y(x), is based on the number of sectored colonies (pink/red and pink/red/white) per 10^3 plated cells on YEPD medium for samples diluted in either H_2O (—■—) or 2 M NaCl (—□—). The spontaneous yield at time 0 (2.8 X 10^{-5}) is based on the occurrence of one pink/red-sectored colony in 36,000 cells tested in the absence of salt.

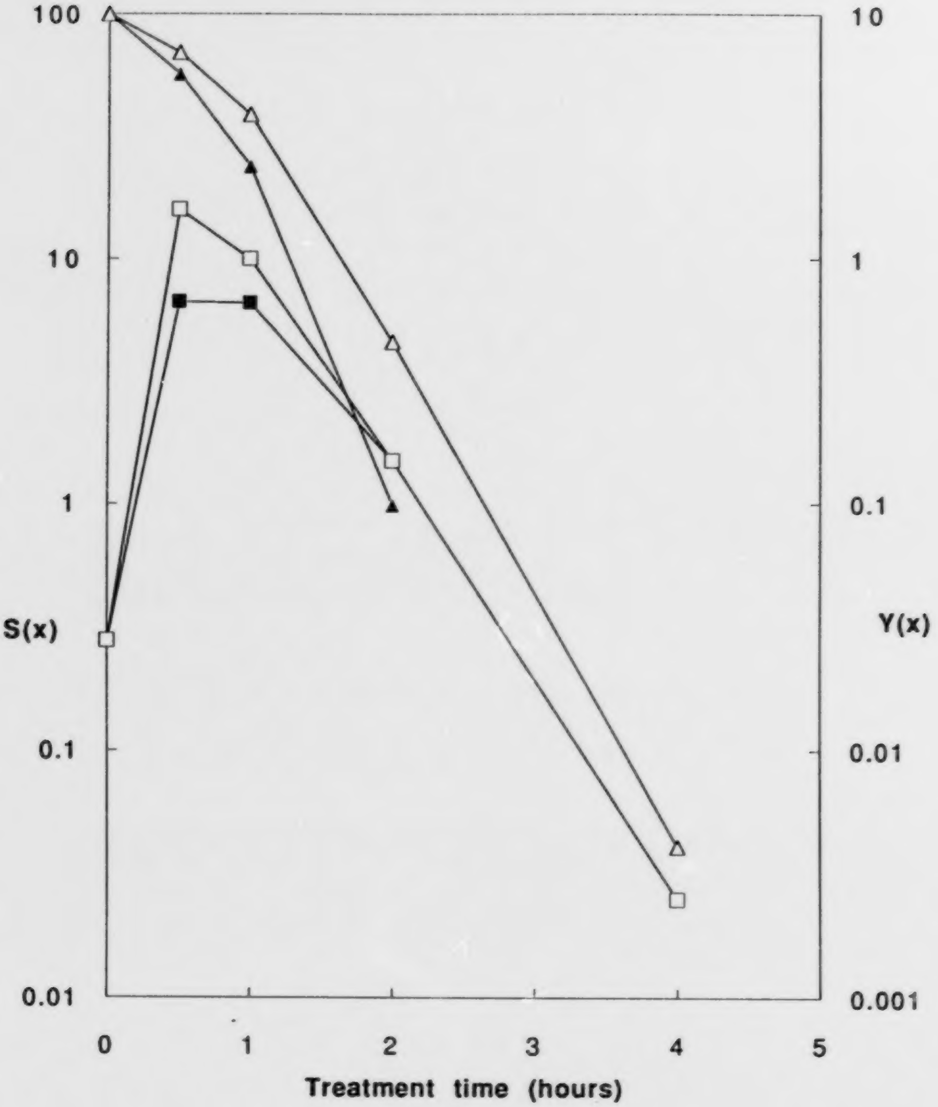

100
10
1
0.1
0.01
S(x)
10
1
0.1
0.01
0.001
Y(x)
0
1
2
3
4
5
Treatment time (hours)

FIGURE 15:

Surviving fraction and yield of conversion at the *ade2* gene of strain D7-144 after treatment with 2 M NaCl. Logarithmic phase cells of yeast strain D7-144 were harvested (at 1.4 X 10^7 cfu/ml, the average from 4 separate experiments) and resuspended in 2.0 M NaCl for up to 4.0 hours. The control group was resuspended in H_2O. Percent survival, S(x), represents the ratio of viable cfu at time x to that of the control at time 0, as determined by colony counts on YEPD plates. Samples treated in NaCl were diluted in either H_2O (—▲—) or 2 M NaCl (—△—) before plating. The yield of conversion at the *ade2* locus, Y(x), is based on the number of colored (pink or red) and white-sectored (pink/white or red/white) colonies per 10^3 plated cells on YEPD medium for samples diluted in either H_2O (—■—) or 2 M NaCl (—□—).

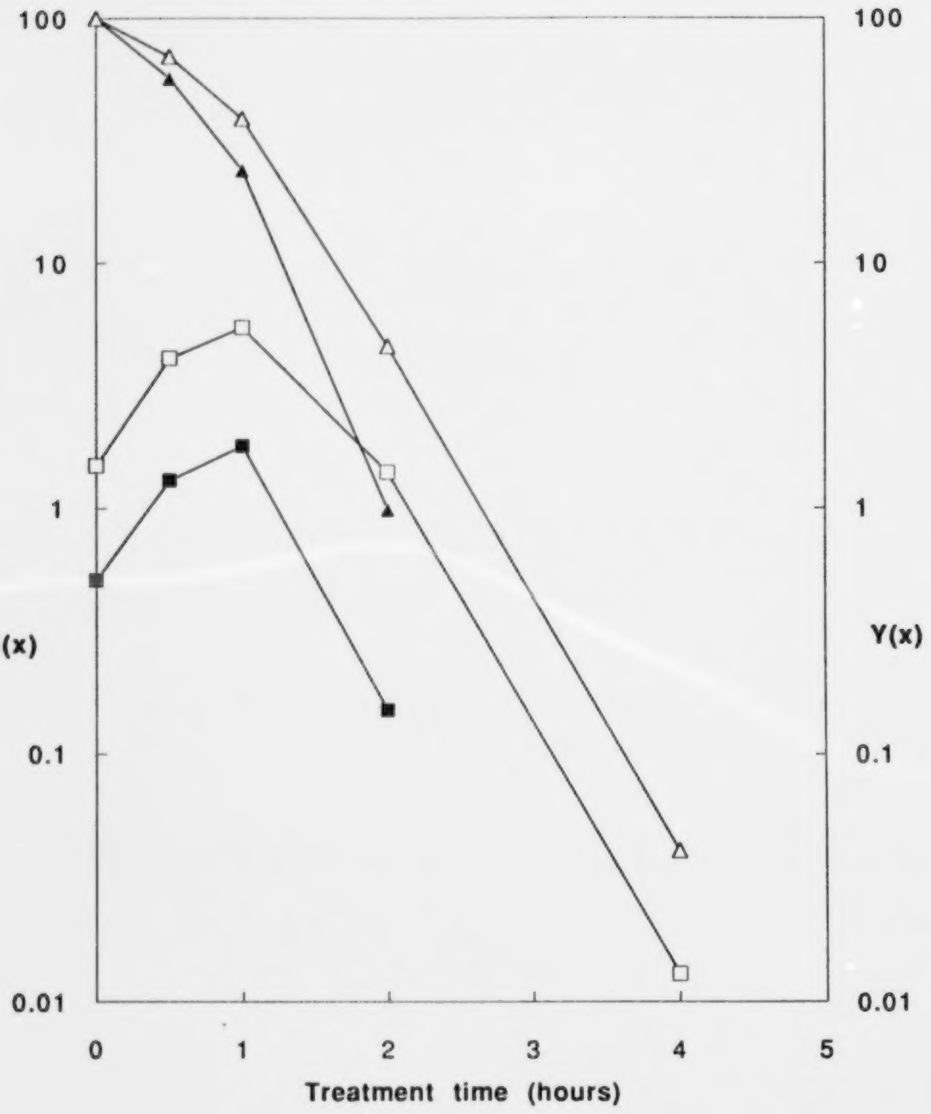
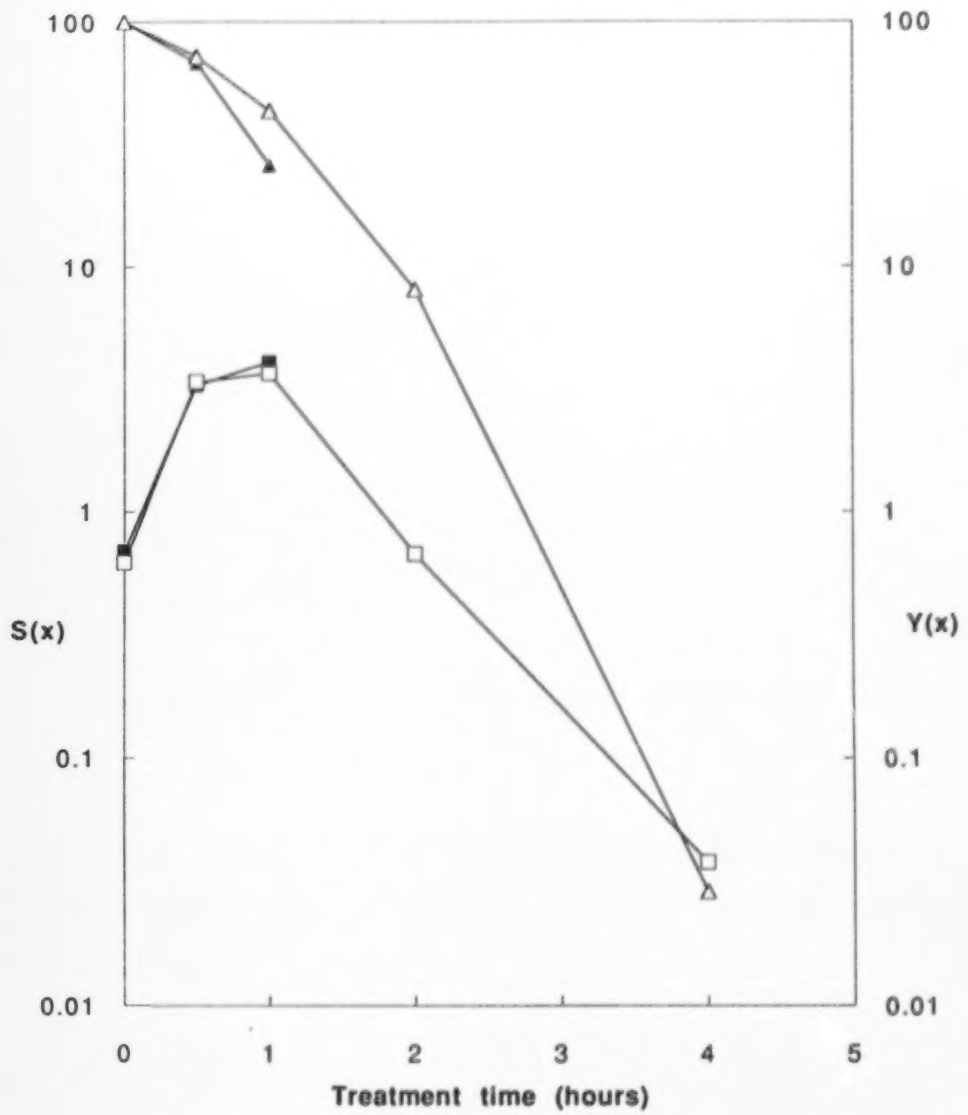

100
10
1
0.1
0.01
S(x)
100
10
1
0.1
0.01
Y(x)
0
1
2
3
4
5
Treatment time (hours)

FIGURE 16:

Surviving fraction and yield of conversion at the *trp5* gene of strain D7-144 after treatment with 2 M NaCl. Logarithmic phase cells of yeast strain D7-144 were harvested (at 1.4 X 10^7 cfu/ml, the average from 4 separate experiments) and resuspended in H_2O (control) or 2.0 M NaCl for up to 4.0 hours. Percent survival, S(x), represents the ratio of viable cfu at time x to that of the control at time 0, as determined by colony counts on YEPD plates. Samples resuspended in NaCl were diluted in either H_2O (—▲—) or 2 M NaCl (—△—) following treatment. The yield of conversion at the *trp5* locus, Y(x), is based on the number of prototrophic colonies per 10^5 plated cells on SC-Trp medium for samples diluted in either H_2O (—■—) or 2 M NaCl (—□—).


100
10
1
0.1
0.01
S(x)
Y(x)
100
10
1
0.1
0.01
0
1
2
3
4
5
Treatment time (hours)

II. Spontaneous Mutation in Yeast Cells which have Adapted to Osmotic Stress

A. Spontaneous mutation rates in hypertonic medium

Reversion rates associated with growth in SC medium supplemented with various salts, chiefly NaCl, were determined using three different procedures for measuring spontaneous mutation rates: the compartmentalization test, the Luria-Delbrück fluctuation test, and the Lassie test. These rates are tabulated in Tables 11, 13 and 15 for alleles *arg4-17*, *his1-7* and *hom3-10* of strain XV185-14C. For compartmentalization tests, the values represent the mean from separate experiments, each consisting of 500-550 compartments. The rates for the Lassie tests represent average values based on the number of plates shown. For the fluctuation test the rates which derive from a single experiment are calculated by two methods, the P(0) method and the method of maximum likelihood (shown in brackets). The latter is based on the probability distribution of the number of mutants in different parallel cultures, as theorized by Lea and Coulson (1949). It will be noticed in Table 11 (and in Tables 13 and 15) that the rates calculated by P(0) for Luria-Delbrück fluctuation tests are often higher and more variable than those calculated by the method of maximum likelihood. This divergence is to be expected if the mean number of mutants per culture, *m*, goes beyond 0.7. For this reason, it is recommended that the fluctuation experiment be designed to obtain values of *m* between 0.3 and 0.7 for optimum efficiency of the P(0) method (Koziol, 1991); otherwise, rate measurements which are based on the method of maximum likelihood are more reliable. Therefore, only the latter values are used in subsequent comparison of rates.

Among the three alleles tested, only reversion at *arg4-17* due to suppressor mutations exhibits an increase in rate for cells growing in SC plus 0.30 or 0.60 M NaCl. In SC medium the spontaneous suppressor mutation rates (*sup*$^-$ → *SUP*$^+$) are 1.5 X 10^{-8}, 1.6 X 10^{-8} and 2.2 (or 2.4) X 10^{-8} based on the three different methods. These rates increase by an average of 2.1-fold to mean values of 3.8 X 10^{-8}, 3.0 X 10^{-8} and 4.8 (or 4.6) X 10^{-8} in SC + 0.30 M NaCl (Table 11). For cells growing in SC +

TABLE 11: Spontaneous reversion rates (*arg4-17* → ARG$^+$) for strain XV185-14C as measured by three different procedures

Compartmentalization Test

Medium (LIM Arg)^a	Cells/ compartment (X 10^{-6})	Number of compartments	Number of compartments bearing revertants		Reversion rate ± SD (x 10^8)	
			SUP$^+$	*ARG4*$^+$	*SUP*$^+$	*ARG4*$^+$
SC	3.49 ±0.21	2197	213	14	1.5 ±0.020	0.13 ±0.013
SC + NaCl 0.30 M	2.70 ±0.32	2174	395	24	3.8 ±1.2	0.20 ±0.62
0.60 M	2.27 ±0.19	2168	391	16	4.3 ±0.62	0.16 ±0.084

Luria-Delbrück Fluctuation Test

Medium	Cells/ml at time of harvest (x 10^{-6})	Number of cells/plate (x 10^{-6})	Residual growth factor	Number of plates	Number of plates bearing revertants		Reversion rate (x 10^8)	
					SUP$^+$	*ARG4*$^+$	*SUP*$^+$	*ARG4*$^+$
SC	28	7.0	1.8	50	17	1	1.7 (1.6)^c	0.082
SC + NaCl 0.30 M	81	20	1.3	47	42	1	4.3 (3.3)	0.042
	54	13	2.3	49	42	1	3.2 (2.6)	0.034
0.60 M	64	10	1.7	50	50	1	— (5.9)	0.055

Lassie Test

Medium (μg/ml Arg)	Number of cells per plate (X 10^{-8})	Number of plates	Total revertants SC (LIM Arg)[a]		Background revertants SC-Arg (OMIS)[b]		Reversion rate (X 10^{8})	
			SUP$^+$	*ARG4*$^+$	*SUP*$^+$	*ARG4*$^+$	*SUP*$^+$	*ARG4*$^+$
SC								
5	4.8 ±0.23	2	43	0	2	0	2.2 ±0.33	—
10	9.2 ±0.18	5	224	6	2	0	2.4 ±0.33	0.12
SC + 0.30 M NaCl								
5	5.6 ±0.11	2	109	1	2	0	4.8 ±0.15	—
10	10 ±0.55	5	485	0	2	0	4.6 ±0.92	—

[a] Growth-limiting concentration of arginine in SC media is 1.0 μg/ml for compartmentalization tests and 5.0 or 10 μg/ml (as specified) for the Lassie plates. Adenine is also reduced to 5.0 μg/ml and 10 or 20 μg/ml respectively to elicit red pigmentation among ARG$^+$ colonies which are not suppressible at *ade2-1*. *SUP*$^+$ = extragenic ochre suppressor; *ARG4*$^+$ = revertant at the ochre nonsense site.

[b] Omission medium is SC without arginine.

[c] Bracketed reversion rates are calculated by the method of maximum likelihood (Lea and Coulson, 1949). All other rates, apart from the Lassie test, are calculated from the proportion of cultures without revertants, where $P(0) = e^{-m}$.

0.60 M NaCl the rates differ between the compartmentalization test (4.3 X 10^{-8}) and the fluctuation test (5.9 X 10^{-8}) so that a further 2-fold effect between 0.30 M and 0.60 M NaCl is not seen. The rate declines from 1.5 X 10^{-8} for cells grown in SC without salt to 5.7 X 10^{-9} for cells grown in SC plus 1.0 M NaCl (Table 12); however, frequency measurements in the previous chapter (Table 6), in which cells underwent one cell division, show no change. Table 11 also includes rates for back mutation at the locus of *arg4-17* (*arg4-17* → *ARG4*$^{+}$) but these values do not show a significant difference between SC and SC + NaCl. Thus, yeast cells which have adapted to growth in hypertonic media exhibit an increased rate of base substitutions of at least one type.

Table 12 shows that KCl, but not sodium saccharin, is also capable of increasing the reversion rates due to suppression. The increase (~1.7-fold) is somewhat less than that caused by NaCl at the same concentrations; however, there is also a 2.3-fold increase in the rate of back mutation at the locus for cells growing in 0.60 M NaCl. Thus KCl, which is an essential electrolyte in all cells for the maintenance of electrochemical gradients across the membrane, is seen here to be causing mutations similar to those caused by NaCl at concentrations that are well within the range of tolerance for growth of yeast cells.

Reversion rates at *his1-7* as measured by compartmentalization tests and those determined by Luria-Delbrück fluctuation tests appear to be discrepant (Table 13). In the presence of NaCl all of the rates determined by compartmentalization tests are progressively antimutagenic, dropping to as much as 6-fold (for SC + 1.0 M NaCl) below spontaneous reversion rates without salt; and, as noted above, there is uncertainty in the declining rate of suppression at *arg4-17* for 1.0 M NaCl. These discrepancies could be explained by sensitivity of the revertants (*HIS1*$^{+}$ or *SUP*$^{+}$) to the salt concentrations present in the compartments. Thus, when histidine or arginine becomes limiting in the compartmentalization tests both non-revertants and osmotically-sensitive revertants cease growth, and only the salt-resistant revertants continue to grow as colonies. In order to check for this possibility, a collection of *HIS1*$^{+}$ revertants generated in SC without

TABLE 12: Effects of other ionic solutes on spontaneous reversion rates (*arg4-17* → ARG⁺) for strain XV185-14C

Compartmentalization Test

Medium (LIM Arg)[a]	Cells/ compartment ($X\ 10^{-6}$)	Number of compartments	Number of compartments bearing revertants		Reversion rate ± SD ($x\ 10^{8}$)	
			SUP⁺	*ARG4⁺*	*SUP⁺*	*ARG4⁺*
SC	3.49 ±0.21[b]	2197	213	14	1.5 ±0.020[b]	0.13 ±0.013
SC + KCl						
0.30 M	2.90 ±0.074	1092	146	5	2.5 ±0.25	0.079±0.025
0.60 M	2.48 ±0.25	1088	145	16	2.8 ±0.81	0.30 ±0.028
SC + Sodium Saccharin						
0.10 M	2.73	504	41	4	1.5	0.14
0.30 M	2.01 ±0.028	1090	25	7	0.54±0.049	0.16 ±0.028
SC + NaCl						
1.0 M	1.32	528	9	2	0.57	0.14

[a] Growth-limiting concentration of arginine in SC media is 1.0 µg/ml. Adenine is reduced to 5.0 µg/ml.

[b] Standard deviations for cell counts and reversion rates are based on values obtained from separate tests. Each test consists of 500 to 550 compartments.

TABLE 13: Spontaneous reversion rates (*his1-7* → *$HIS1^+$*) for strain XV185-14C

Compartmentalization Test

Medium (µg/ml His)[a]	Cells/ compartment ($X\ 10^{-6}$)	Number of compartments	Number of compartments bearing revertants	Background revertants per compartment ($X10^3$)	Reversion rate ± SD[b] ($x\ 10^8$)
SC					
(0.2)	1.7 ±0.12	3057	577	2.2 ±1.4	6.0 ±1.3
(0.4)	4.2 ±0.61	4736	2043	2.4 ±1.1	6.7 ±1.1
SC + NaCl (0.2 µg/ml)					
0.15 M	2.1	480	78	1.7	4.3
0.20	1.5	480	61	3.8	4.3
0.30	1.6 ±0.38	930	106	13	3.7 ±0.30
0.46	1.7	480	44	1.6	2.8
1.0	0.88	480	11	1.2	1.2
(0.4 µg/ml)					
0.30 M	4.5	552	186	1.1	4.5
0.60	3.4	1104	132	1.0	1.9 ±0.040

Luria-Delbrück Fluctuation Test

Medium	Cells/ml at time of harvest (x 10^{-6})	Number of cells/plate (x 10^{-6})	Residual growth factor	Number of plates	Number of plates bearing revertants	Reversion rate (x 10^{8})
SC	28	7.0	1.3	50	37	7.3 (6.1)[c]
	23	3.8	1.7	50	28	6.5 (6.1)
SC + NaCl						
0.30 M	81	20	1.2	47	47	— (6.3)
	54	13	2.0	49	47	6.0 (4.9)
0.60 M	64	10	1.6	50	48	9.3 (6.6)

[a] Growth-limiting concentrations of histidine used in SC media for compartmentalization tests are 0.2 or 0.4 µg/ml as specified in parentheses.

[b] Standard deviations for parameters measured in compartmentalization tests are based on values obtained from independent tests. Each test consists of 500-550 compartments.

[c] Bracketed reversion rates are calculated from the method of maximum likelihood (Lea and Coulson, 1949). All other rates are calculated from the proportion of cultures without revertants, where $P(0) = e^{-m}$.

TABLE 14: Effect of NaCl on growth of HIS$^+$ revertants obtained from SC medium

Test plate	Number of revertants showing growth[a]		Total
	ρ^+	ρ^-	
YD	69	18	87
SC-His	69	18	87
YD (1.0 M NaCl)	69	17	86
SC-His (0.6 M NaCl)	49	13	62
SC-His (1.0 M NaCl)	0	0	0
SC+His (1.0 M NaCl)	68	0	68

[a] Number of revertants that grow on each test plate among 87 different ρ^+ and ρ^- colonies obtained from SC medium

salt was tested on SC agar medium supplemented with NaCl to determine whether a portion of the revertants would not grow. Among 87 different colony isolates tested (Table 14), 29% (25/87) show weak growth on SC-His + 0.60 M NaCl and all of the isolates exhibit reduced or no growth on SC-His + 1.0 M NaCl. With respect to osmotic sensitivity of nonsense suppressors, Singh (1977) reports that growth inhibition of various suppressors (including all of the tyrosine-inserting suppressors) on hypertonic medium (1.5 M KCl, 2.5 M ethylene glycol) is a function of suppressor efficiency; and Liebman and Sherman (1976) note that both low and high-efficiency suppressor alleles are possible for any single suppressor gene, but it is often the high efficiency form that is osmotically sensitive. Taken together, these data suggest that antimutagenesis by salt as revealed by the compartmentalization tests in Tables 12 and 13 can be explained as a response of the gene product rather than a response of the DNA. At these dose ranges the Luria-Delbrück fluctuation test provides the more reliable measure of reversion rate because it rescues the osmotically-sensitive revertants from the hypertonic growth medium to a nonhypertonic agar plate.

Reversion rates at *his1-7*, as calculated by the method of maximum likelihood in the fluctuation tests, are constant at 6.1 to 6.6 X 10^{-8} with or without inclusion of NaCl in the medium (Table 13). Since the reversion of both *his1-7* and *arg4-17* comes about by base substitutions, the inability to detect an increase at both of these alleles by growth in salt-supplemented media could be attributed to one of several possibilities: (1) NaCl reduces the efficiency of suppression at upper salt concentrations (as described above) but increases the efficiency at lower concentrations. Thus, salt may not be contributing to extra mutations; rather it improves the efficiency of suppression in SC + 0.30 M or 0.60 M NaCl (or KCl) beyond that which is seen for SC medium without salt; (2) The increase in the rate of suppression at *arg4-17* is caused by an enhanced number of base substitutions at sup loci which are not well represented among the types of missense mutations that revert *his1-7*; or (3) NaCl induces a rearrangement of the spectrum of base substitutions so that some types of mutations are increased while others are reduced. The net effect of these changes upon the *his1-7* locus restores the same reversion rate. These three possibilities could be resolved by a forward mutation assay from which it is possible to obtain an overall forward mutation rate for base substitutions and a spontaneous mutation spectrum through DNA sequencing.

Reversion rates for the frameshift allele, *hom3-10*, are tabulated in Table 15, and these values are plotted in Figure 17 against the cell concentration at which the rates were measured. The graph shows that reversion rates increase as a function of cell concentration, and that generation of these types of frameshift mutations is independent of the inclusion of salt in the medium. Possible explanations for the interdependence of reversion rate and cell concentration are as follows: (1) The growth rate of *HOM*$^+$ revertants exceeds that of *hom3*$^-$ cells; or, more likely, *HOM*$^+$ revertants are selected if either threonine or methionine becomes growth-limiting. (2) The overall frameshift reversion rate (or, by a directed mechanism, only the reversion rate at *hom3-10*) increases as a function of time after the cells have become stressed by starvation for threonine and/or methionine, or (3) There is no starvation stress. For reasons that are not known, the cells exhibit a concentration-dependent increase in the rate of frameshift mutations. For hypotheses 1 and 2 the onset of the reversion rate increase is restricted to a late cell concentration when amino acids become limiting, but for hypothesis 3 the reversion rate increases as a continuous function of the cell concentration. The few points plotted in Figure 17 and the large variance associated with the rates at the lower cell concentrations preclude an absolute distinction between a quadratic, sinusoidal or linear function for these data. However the possibilities could be narrowed down if a forward mutation assay showed that frameshift mutations comprise a larger fraction of the mutation spectrum with increase in cell concentration.

In improvising a reproducible method of measuring forward mutation rates at the *URA3* gene, it was observed that the yield of *ura3*$^-$ mutants could be considerably improved by allowing for phenotypic lag. Table 16

TABLE 15: Spontaneous reversion rates (*hom3-10* → HOM+) for strain XV185-14C

Compartmentalization Test

Medium (LIM Met)[a]	Cells/ compartment (X 10^{-6})	Number of compartments	Number of compartments bearing revertants	Background revertants per compartment (X10^{3})	Reversion rate ± SD[b] (x 10^{8})
SC	19 ± 11	2206	582	0.39 ± 0.18	0.84 ± 0.10
SC + NaCl					
0.30 M	15 ± 8.9	2052	548	0.38	1.2 ± 0.28
0.60 M	5.1 ± 0.18	1104	144	0.42	0.67 ± 0.29

Luria-Delbrück Fluctuation Test

Medium	Cells/ml at time of harvest (x 10^{-6})	Number of cells/plate (x 10^{-6})	Residual growth factor	Number of plates	Number of plates bearing revertants	Reversion rate (x 10^{8})
SC	28	7.0	2.4	50	16	1.2 (1.0)[c]
	69	11	1.4	50	34	3.6 (2.6)
SC + NaCl						
0.30 M	81	20	1.0	47	40	4.8 (3.4)
	54	13	2.2	50	36	2.2 (1.7)
0.60 M	64	10	1.5	50	27	2.4 (2.4)

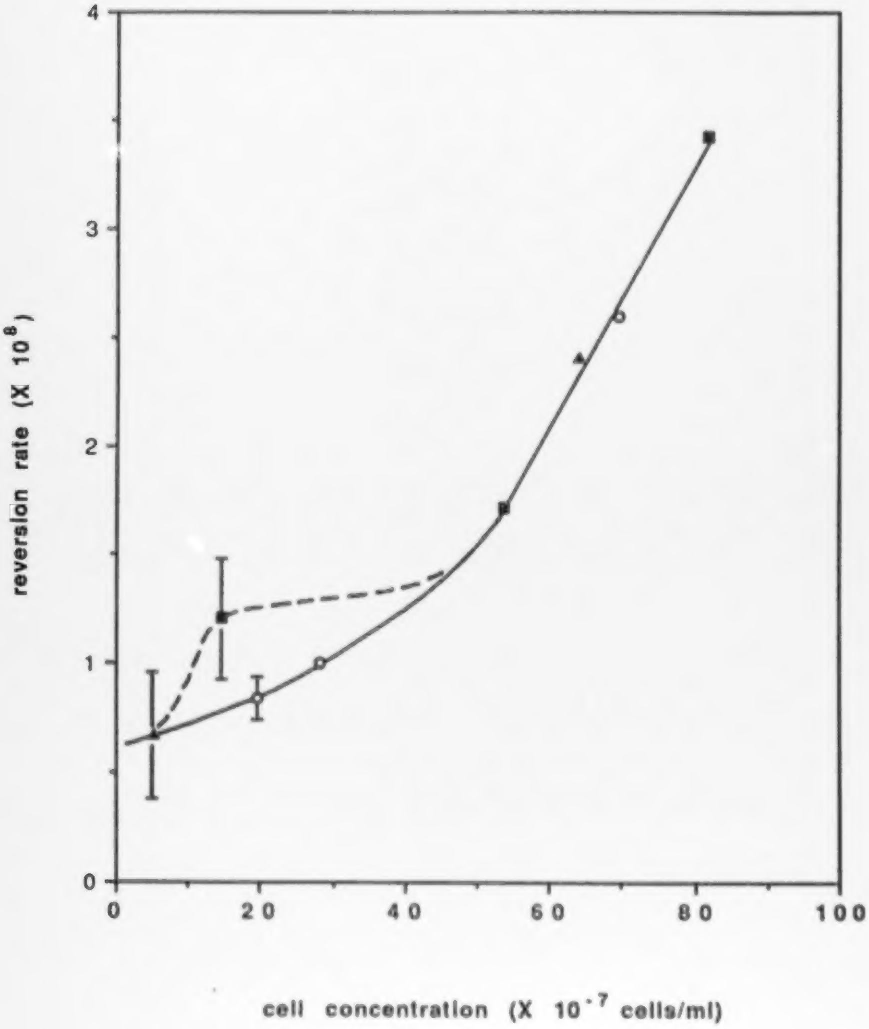
[a] Growth-limiting concentration of methionine as used in SC media for compartmentalization tests is 0.5 μg/ml.

[b] Standard deviations for cell counts, background reversion frequencies and reversion rates in compartmentalization tests are based on values obtained from separate tests. Each test consists of 500 to 550 compartments.

[c] Bracketed reversion rates are calculated from the method of maximum likelihood (Lea and Coulson, 1949). All other rates are calculated from the proportion of cultures without revertants, where $P(0) = e^{-m}$.

FIGURE 17:

Frameshift reversion rate as a function of cell concentration with or without addition of NaCl to the growth medium. Reversion rates at *hom3-10* of haploid strain XV185-14C and the corresponding cell concentrations at which these rates were measured are from Table 15. Rates with standard deviation bars derive from compartmentalization tests; all others are single measurements from Luria-Delbrück fluctuation tests. Addition of NaCl to the growth medium is symbolized as follows: o, SC without NaCl; ■, SC + 0.30 M NaCl; ▲, SC + 0.60 M NaCl.



4
3
2
1
0
reversion rate (X 10^8)
0
20
40
60
80
100
cell concentration (X 10^{-7} cells/ml)

TABLE 16: Effect of growth in SC-ura on recovery of FOA-resistant colonies

Treatment conditions	Recovery time in SC-ura	Mutation yield[b] ($X 10^7$)			Total FOA^R colonies
Hours[a]	Hours	URA^+	*ura5⁻*	*ura3⁻*	
H_2O 0.0	0	2.1 (3)	0.72(1)	2.1 (3)	7
	7	6.3 (4)	—	110 (15)	19
7.5	0	2.1 (4)	0.53(1)	0.53(1)	6
	15	0.96(2)	—	350 (26)	28

Treatment conditions[c]	Recovery time in SC-ura	Mutation rate[d] ($X 10^7$)		Number of plates
	Hours	URA^+	*ura3⁻*	*(N)*
2% Dextrose	0	5.6 (36)	0.77 (8)	50
	7-15	0.47(25)	1.6 (15)	49

[a] Cells of haploid strain ES55-6A (grown in YEPD to $1.7 X 10^7$ cfu/ml) were centrifuged, resuspended in H_2O, and plated to SC+FOA either with or without growth in SC-ura beforehand. In the second experiment the cells were held in H_2O for 7.5 hours prior to the recovery in SC-ura.

[b] Bracketed numbers represent the number of FOA^R colonies as characterized by complementation into one of 3 groups.

[c] Cells of haploid strain XV185-14C (grown in SC-ura to $3.5 X 10^7$ cfu/ml) were used to inoculate 50 tubes of SC medium each commencing with a cell concentration of $1.5 X 10^3$ cells/ml. Following cell division to an average cell concentration *(C)* of $6.9 X 10^7$ cells/ml, the cells were centrifiged, resuspended in 2% dextrose, and plated to SC+FOA with or without growth in SC-ura beforehand.

[d] Bracketed numbers represent the number of plates bearing 1 or more FOA^R colonies characterized as either *ura3⁻* or *$URA3^+$* by complementation.

shows the effect of growing the cells in SC-ura for 3 or more generations prior to plating on SC+FOA. Calculation of mutation yields and rates for ura$^-$ cells are based on the cell concentration after dilution in SC-ura (i.e., before cell division in SC-ura); whereas, the same values for URA$^+$ (for$^-$) cells, which can undergo division in this medium, are based on the cell concentration after growth in SC-ura. The data show that the yield of *ura3*$^-$ FOA^R colonies for the haploid strain ES55-6A is significantly increased (2.1 → 110 and 0.53 → 350 per 10^7 plated cells) when the cells are grown in SC-ura. In contrast, the Luria-Delbrück fluctuation test with strain XV185-14C (shown in the lower half of Table 16) reveals a much smaller effect (0.77 → 1.6 *ura3*$^-$ mutations per 10^7 cells per generation). The basis for this difference is not clear, except that in the experiments with ES55-6A the cells were resuspended in H_2O before recovery in SC-ura, unlike the treatment of XV185-14C where the supply of glucose was maintained. The interpretation by Lee et al., 1988 of an improved recovery of *ura3*$^-$ colonies following growth in uracil-deficient medium is that residual growth of *ura3*$^-$ cells is needed to deplete the cytoplasmic pool of OMP decarboxylase encoded by the *URA3*$^+$ gene after the occurrence of a mutation. Presumably, a lack of uracil stimulates expression of the enzymes required for *de novo* synthesis (cf. pyrimidine salvage). However, the data in Table 16 for strain XV185-14C do not support this hypothesis. Instead, one is led to believe that depletion of glucose in the medium (e.g., by resuspending the cells in H_2O) might be a significant factor in the expression of mutations at the *URA3* gene. Other experiments with strain ES63-11A indicate that recovery of FOA^R colonies is diminished if growth in SC-ura is prolonged beyond say 24 hours (data not shown). Therefore, the period of growth subsequent to dilution in SC-ura had to be closely monitored, by taking cell counts, to allow for no more than 3 generations. In summary, it is apparent from these data that certain factors may influence the formation of *ura3*$^-$ mutations, apart from phenotypic lag which could affect the recovery of *ura3*$^-$ mutants.

Table 17 summarizes the mutation rates at the *URA3* gene as determined from Luria-Delbrück fluctuation tests. These values are

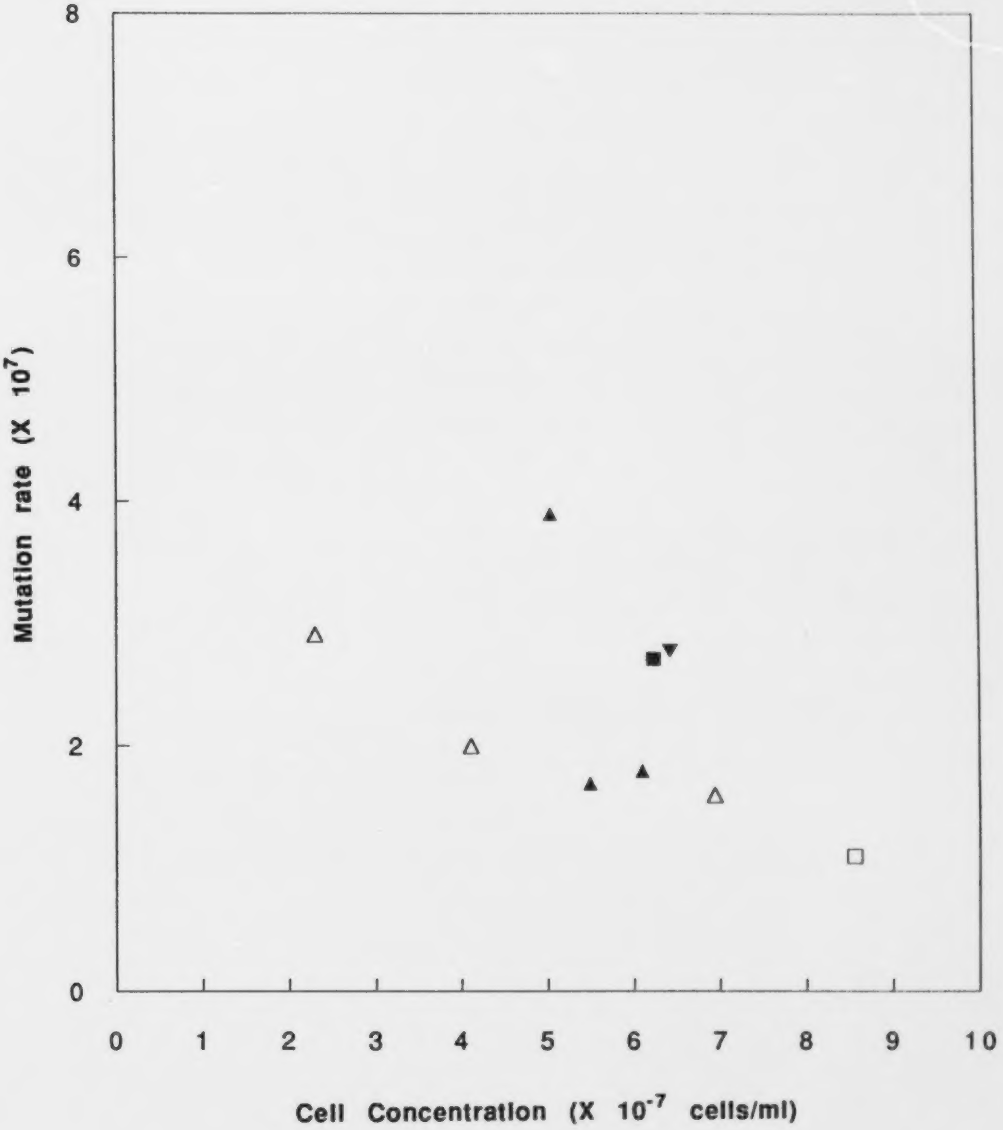
TABLE 17: Forward mutation rates (*URA3*$^+$ → *ura3*$^-$) for strain XV185-14C as determined by Luria-Delbrück fluctuation tests

Medium	Cells/ml at time of harvest (x 10^{-6})	Number of cells/plate (x 10^{-6})	Number of plates	Number of plates bearing *ura3* mutants	Mutation rate[a] (x 10^{8})
SC	23	0.57	50	14	29 (26)
	41	0.68	50	12	20 (18)
	69	1.1	49	15	16 (15)
SC + NaCl 0.30 M	50	0.83	40	19	39 (34)
	55	0.91	50	13	17 (16)
	61	1.0	50	15	18 (17)
0.60 M	64	1.1	50	22	28 (24)

[a] Mutation rates are calculated by the P_0 method (unbracketed values) and by the method of maximum likelihood (bracketed).

FIGURE 18:

Effect of cell concentration on forward mutation rate, *URA3* → *ura3*$^-$. Forward mutation rates at *URA3* and the corresponding cell concentrations at which these rates were measured are taken from Table 17 for strain XV185-14C. Addition of NaCl to the growth medium is symbolized as follows: Δ,❑ = SC without NaCl; ▲,■ = SC + 0.30 M NaCl; ▼ = SC + 0.60 M NaCl. (triangles correspond to strain XV185-14C; squares represent ES63-11A).


Mutation rate (X 10^7)
0
2
4
6
8
0
1
2
3
4
5
6
7
8
9
10
Cell Concentration (X 10^{-7} cells/ml)

plotted against cell concentration in Figure 18. The graph shows that forward mutation rates decline slightly when the cells are harvested at concentrations approaching stationary phase, unlike the reversion rates in Figure 17. The decline might be attributed to selection in that production of mutations in the *URA3* gene could cause at least some delay while enzyme activities adjust to an altered source of metabolites in the maintenance of pyrimidine nucleotide pools (i.e., all UTP and CTP in *ura3*$^-$ cells must now derive from exogenous uracil). Alternatively, the decline could represent a reduction in the survival of *ura3*$^-$ mutants that were formed earlier in growth. This situation would prevail if uracil, though supplemented at 20 μg/ml in SC medium at the outset of growth, became depleted. Since half of the recoverable mutations would accrue to the last cell division and there is little more than a 2-fold decline in the forward mutation rates at upper cell concentrations, it is likely that forward mutation rates at the *URA3* gene, without supplementation of NaCl to the medium, are relatively constant.

In the presence of NaCl, spontaneous mutation rates are not significantly affected (Figure 18). The average mutation rate at the *URA3* gene in the absence of osmotic stress, based on 4 rate measurements, is 1.9×10^{-7}. In the presence of 0.30 to 0.60 M NaCl the mean is 2.6×10^{-7}, based on 5 measurements. The average cell concentrations upon which these rates are based are nearly the same at 5.5×10^{7} and 5.9×10^{7}, respectively. On this basis, two forward mutation rates are compared in Tables 18 (cells grown without salt) and 19 (cells grown with NaCl). All of the *ura3*$^-$ mutants obtained by these growth conditions have been sequenced, so that a rate determination for each type of mutation can be deduced as a fraction of the composite rate.

B. Spontaneous mutation spectra

Growth of yeast cells in 0.30 or 0.60 M NaCl causes rearrangement of the mutation spectrum. The spontaneous mutation spectra for three different strains of yeast are shown in Table 18. Although only 25 to 35 mutations were sequenced for each

TABLE 18: Spontaneous mutation spectra for *ura3* mutations obtained from yeast cells grown in SC or YD

Mutation type	Yeast strain (growth medium)			Combined total	Rate ($\times 10^8$)
	XV185-14C (SC)	QW6-2A (SC)	ES63-11A (YD)		
Base substitutions:					
Transitions					
G•C → A•T	3	4	3	10	2.1
A•T → G•C	3	5	1	9	1.9
Transversions					
G•C → T•A	9	10	6	25	5.3
G•C → C•G	6	4	3	13	2.8
A•T → C•G	4	3	3	10	2.1
A•T → T•A	3	1	1	5	1.1
Total	28	27	17	72	15
Deletions and insertions:					
Small frameshifts					
− G or C		1	1	2	0.43
− A or T	3	1	5	9	1.9
− 2 or 3 bp	2		1	3	0.64
+ A or T	1		1	2	0.43
+ 2 or 3 bp			1	1	0.21
Large deletions				0	< 0.21
Total	6	2	9	17	3.6
TOTAL	34	29	26	89	19[a]

[a] A mutation rate of 1.9×10^{-7} mutations/cell/generation corresponds to a cell concentration of 5.5×10^{7} cells/ml from Figure 18. Rates for each type of alteration are fractions of this total rate.

TABLE 19: Spontaneous mutation spectra for *ura3* mutations obtained from yeast cells grown in SC supplemented with 0.30 M or 0.60 M NaCl

Mutation type	Growth medium		Combined total	Rate (X 10^8)
	SC + 0.30 M NaCl	SC + 0.60 M NaCl		
Base-pair substitutions:				
Transitions				
G•C → A•T	6	4	10	4.7
A•T → G•C	0	1	1	0.47
Transversions				
G•C → T•A	15	8	23	11
G•C → C•G	1	0	1	0.47
A•T → C•G	2	0	2	0.95
A•T → T•A	4	2	6	2.8
Total	28	15	43	20
Deletions and insertions:				
Small frameshifts				
− G or C	1	0	1	0.47
− A or T	3	0	3	1.4
− 2 or 3 bp	3	2	5	2.4
+ A or T	0	1	1	0.47
+ 2 or 3 bp			0	< 0.47
Large deletions	1	0	1	0.47
Total	8	3	11	5.2
TOTAL	36	18	54	26[a]

[a] A mutation rate of 2.6 X 10^{-7} mutations/cell/generation is based on the mean of 5 estimates, as shown in Figure 18. These correspond to an average cell concentration (at time of harvest) of 5.9 X 10^{7} cells/ml. Rates for each type of alteration are fractions of this total rate.

strain, the relative frequency of the 6 types of base substitutions is quite consistent. Transversions represent 74 per cent (53/72) and transitions 26 per cent (19/72) of the total base substitutions, which is close to the expected frequencies for random occurrence of these two classes of mutation. However, it is unlikely that the distribution is random as indicated by the transversions, where one class, G•C→T•A, is consistently more frequent than the other 3 classes (especially A•T→T•A). Most (72/89 = 81 per cent) of the total mutations sequenced are base substitutions; however, frameshift mutations occupy a variable fraction of the total among the different strains, ranging from 7 (2/29) to 35 (9/26) per cent.

The mutation spectra for yeast strain XV185-14C grown in SC supplemented with 0.30 or 0.60 M NaCl are presented in Table 19. Yeast cells which have adapted to growth under these conditions show little change in the rates of both base substitutions and frameshift mutations (2.0×10^{-7} and 5.2×10^{-8}, respectively) relative to the rates for growth without NaCl (1.5×10^{-7}; 3.6×10^{-8}). A comparison of the rates for the six types of base substitutions indicates that NaCl has caused, either directly or indirectly, a substantial rearrangement of the mutation spectrum. There is a 2-fold increase of base substitutions that yield replacement with an A•T base pair and a 3- to 4-fold decrease among those that produce replacement with G•C. This high specificity for substitution by A or T is of interest because these base substitutions have been shown to be the most frequent mutation resulting from replication across from AP sites (Tessman, 1976; Strauss et al., 1982).

As a further measure of the effect of NaCl on the relative distribution of base substitutions, missense reversions at *his1-7* were distinguished as feeders and nonfeeders (see Figure 19), wherein the types of base substitutions represented by these two classes of intragenic suppressor are not the same. *HIS1*$^{+}$ revertants of both types have been sequenced (Table 20). These data confirm that reversion of the missense allele, *his1-7*, is largely due to intragenic suppression by second-site mutations within the open reading frame of the gene. Only one of 37 revertants was due to back mutation at position 798 through

FIGURE 19:

Phenotypes of feeder and nonfeeder revertants on SC-His agar medium. The plates depict 5 nonfeeder-type revertants and 6 feeders, 2 of which show weak overexpression of histidine.

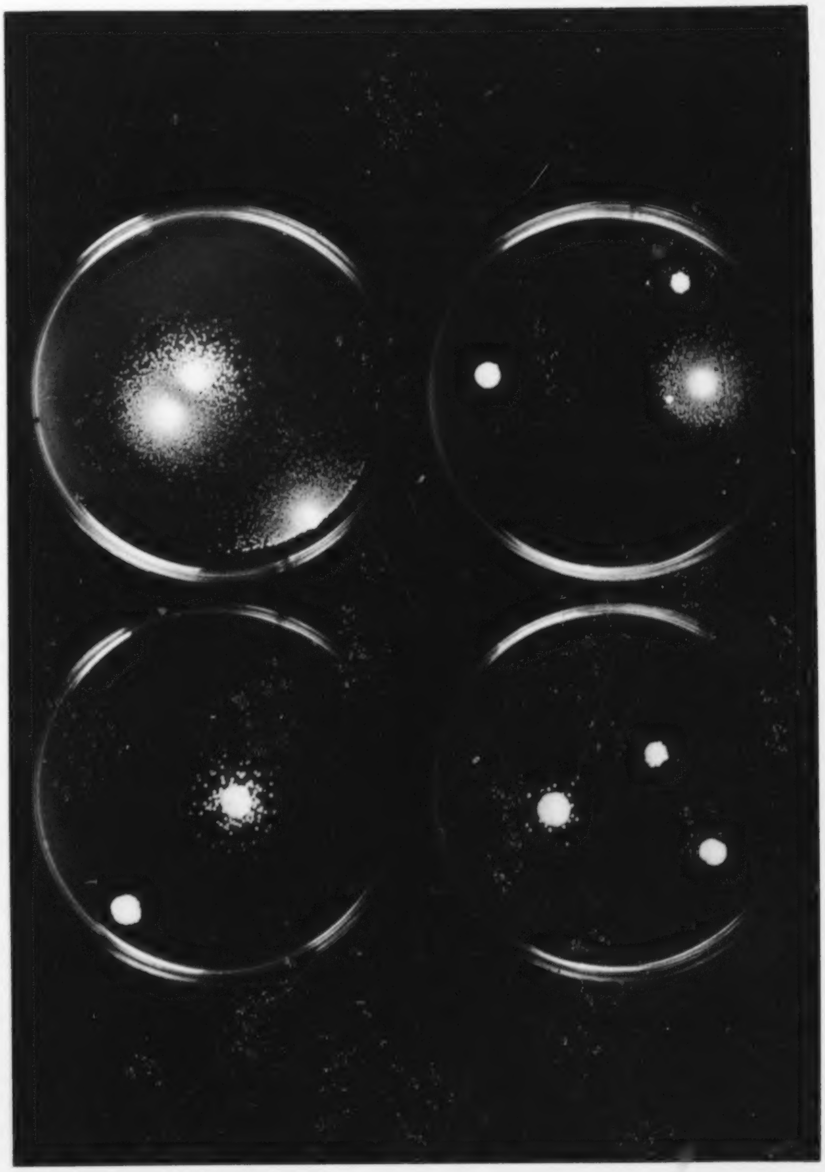


TABLE 20: Types of base pair substitutions induced by reversion of missense allele *his1-7*

Mutation type	Feeder-type revertants	Nonfeeders
Transitions		
G•C → A•T	6	6
A•T → G•C	0	5
Transversions		
G•C → T•A	6	4
G•C → C•G	2	2
A•T → C•G	2	2
A•T → T•A	2	0
Total	18	19

TABLE 21: Reversion rates (*his1-7* → HIS1$^+$) for intragenic suppressors of *his1-7*

Medium		Reversion rates[a] (X 10^8)		
		Feeder-type revertants	Nonfeeders	Total
SC		1.2	4.8	6.1
		1.3	4.8	6.1
	$\overline{X}$	1.3 (0.21)	4.8 (0.79)	6.1
SC + NaCl		1.6	4.7	6.3
0.30 M		1.3	3.6	4.9
	$\overline{X}$	1.4 (0.25)	4.2 (0.74)	5.6
0.60 M		2.0 (0.30)	4.6 (0.70)	6.6

[a] All rates are based on the method of maximum likelihood. Bracketed numbers indicate the fraction of the total rate of reversion.

transition from A (encoding isoleucine) to G (methionine). Table 19 shows that most (14/18 = 78%) of the mutations that give rise to feeders involve substitution by A•T; whereas only half (10/19 = 53%) of the mutations among the nonfeeders are of this type. When the reversion rates in Table 13 are subdivided to include the observed proportion of feeder-type colonies among the *HIS1*$^+$ revertants, the results in Table 21 show an increase for feeder-type revertants but not nonfeeders. The increase in the reversion rate of feeders is only 1.5-fold (1.3 → 2.0 X 10^{-8}), which compares with 2-fold for reversion at *arg4-17* (from Table 11). If the frequency of substitutions by A or T relative to G or C shows rearrangement as in the *URA3* gene, then the net increase in reversion rate for the feeders could be attributed to the larger fraction of opportunities for substitution by A or T within this group. These data therefore support the notion that the increase in specificity for substitution by A•T base pairs is approximately balanced by a decrease in specificity for substitution by G•C.

The rate of frameshift mutations is directly proportional to cell density. The tabulation of forward frameshift mutation rates at the *URA3* gene in Table 22 indicates that the rates increase by 4.3-fold from 1.4 to 6.0 X 10^{-8} as the cell concentration is increased 23-fold from 1.0 X 10^7 to 2.3 X 10^8 cells/ml. This compares with a 5.1-fold increase from 0.67 X 10^{-8} to 3.4 X 10^{-8} for the rate of reversion at *hom3-10*, when the cell concentration ranges 16-fold from 5.1 X 10^6 to 8.1 X 10^7 (Table 15). Therefore, it is apparent from both the reversion and forward mutation data that frameshift mutations are produced more frequently as the population becomes more dense.

The rates as estimated in Tables 18 and 19 may require adjustment if the combined totals are taken from different samples or strains that were not evaluated at the same cell concentration. In Table 18, the proportion of frameshift mutations among the combined total of 89 mutations (17/89 = 19%) is somewhat greater than the expected proportion for a cell concentration of 5.5 X 10^7 cells/ml, upon which the rates are based. A closer approximation would be 15.8% which allows for 14 frameshift mutations in a total of 86. In Table 19, the

TABLE 22: Effect of cell density on the rate of production of frameshift mutations at the *URA3*$^+$ locus

Medium	Cells/ml at time of harvest ($X 10^{-6}$)	Proportion of frameshifts among total mutations	Frameshift mutation rate ($X 10^{8}$)
SC	10	0.069 (2/29)	1.4
	41	0.077 (1/13)	1.5
	69	0.24 (5/21)	3.8
YD	230	0.35 (9/26)	6.0

proportion of frameshift mutations observed among the *ura3*⁻ mutants obtained from 0.30 M NaCl at a cell concentration of 5 X 10^7 cells/ml is 22% (8/36) and the additional data from 0.60 M NaCl does not cause a significant change (11/54 = 20%). Thus, adjustments to the rate measurements in Tables 18 and 19 are relatively minor, at least in so far as these tables have been set up with standardization to a cell concentration of 5.5 X 10^7 cells/ml.

The growth of yeast cells in NaCl has increased the rate of frameshift mutations by 1.7-fold (assuming the overall rate of deletions and insertions in Table 18 is closer to 3.0 X 10^{-8}), largely due to deletions involving 2 or more base pairs. Single-base insertions or deletions show no change in rate, in keeping with previous observations that frameshift reversions at *hom3-10* are not influenced by the presence of NaCl. Most of these reversions are brought about by single base insertions at suppressor tRNA genes (Wang et al., 1990; Wang et al., 1991).

The presence of NaCl in the medium alters the base substitutions within the N-terminal but not the C-terminal half of the *URA3* coding region. The *ura3*⁻ mutations as sequenced by the dideoxy chain termination method are presented in Appendices 3 to 6. These mutations are also grouped according to whether they occur in the N-terminal or C-terminal half of the gene in Tables 23 and 24. The spontaneous base substitutions and frameshift mutations obtained from cells which were grown in SC or YEPD (Table 23) occur mostly in the N-terminal half of the coding region (51/89 = 57%). Among 25 G•C→T•A or C•G→A•T transversions most (19/25 = 76%) occur in the C-terminal half of the coding region (Table 24), which thus contribute to half (19/38 = 50%) of the mutations in this part of the gene. Thus, 26 out of 34 base substitutions (76%) in the C-terminal half of the gene exhibit specificity for substitution by A or T. Since the GC-content for the two halves of the coding region is essentially the same (180/400 = 45% vs. 186/400 = 46.5%) these data suggest that some form of mutational preference is acting across the span of the coding region ($\chi^2 = 9.5$, $p < 0.005$). The mutational specificity for base substitutions in

TABLE 23: Summary of mutations sequenced at the *URA3* gene

Region of *URA3* gene	Mutation[a]	Mutation rate[b] (X 10^8)	
		SC or YD	SC + NaCl
N-terminal half	→ G or C	5.1 (24)	0.95 (2)
	→ A or T	3.0 (14)	10 (22)
	−1/ +1/ Δ	2.8 (13)	2.4 (5)
		11 (51)	14 (29)
C-terminal half	→ G or C	1.7 (8)	0.95 (2)
	→ A or T	5.6 (26)	8.1 (17)
	−1/ +1/ Δ	0.85 (4)	2.8 (6)
		8.1 (38)	12 (25)

[a] Mutations are grouped into 3 categories: base substitutions to G or C (→ G or C), base substitutions to A or T (→ A or T), and frameshift mutations, deletions or insertions (−1/ +1/ Δ).

[b] The rates for each type of alteration are based on the average value (1.9 X 10^{-7}) from 4 estimates of mutation rates, as plotted in Figure 18, for cells cultured in SC or YD and 5 estimates for SC + NaCl (2.6 X 10^{-7}). These rates correspond to mean cell concentrations of 5.5 X 10^7 and 5.9 X 10^7 cells/ml. The numbers in brackets represent the actual number of mutations sequenced.

TABLE 24: Summary of base substitutions sequenced at the *URA3* gene

Region of *URA3* gene	Base substitution[a]	Mutation rate (X 10^8)	
		SC or YD	SC + NaCl
N-terminal half	T•A → C•G	1.7 (8:0)[a]	0.47 (0:1)
	T•A → G•C	1.7 (2:6)	0.47 (0:1)
	C•G → G•C	1.7 (5:3)	— (0)
		5.1 (15:9)	0.95 (0:2)
	C•G → T•A	0.85 (1:3)	1.4 (1:2)
	C•G → A•T	1.3 (3:3)	7.6 (7:9)
	T•A → A•T	0.85 (2:2)	1.4 (1:2)
		3.0 (6:8)	10 (9:13)
C-terminal half	T•A → C•G	0.21 (1:0)	— (0)
	T•A → G•C	0.43 (1:1)	0.47 (1:0)
	C•G → G•C	1.1 (1:4)	0.47 (1:0)
		1.7 (3:5)	0.95 (0:2)
	C•G → T•A	1.3 (1:5)	3.3 (1:6)
	C•G → A•T	4.1 (9:10)	3.3 (6:1)
	T•A → A•T	0.21 (0:1)	1.4 (1:2)
		5.6 (10:16)	8.1 (8:9)
		15 (34:38)	20 (18:25)

[a] The left nucleotide of each base pair corresponds to the nontranscribed strand of DNA. Bracketed ratios after each rate represent the number of mutations involving pyrimidines versus purines in the nontranscribed strand.

the N-terminal half is of the opposite type, where 24 out of 38 base substitutions (63%) give rise to G or C residues ($\chi^2 = 1.32$, $p < 0.3$).

The mutational preference as described could be due, in part, to the occurrence of hot spots, which refer to the tendency of a specific mutation to recur at one site. The data in Appendix 3 reveals two hot spots: a C•G→A•T transversion at position 605 which occurred 4 times among 89 mutations and a G•C→T•A transversion at position 340 which occurred twice. Taking this in to consideration the indication of mutational preference for substitution by A or T in the latter half of the gene appears to be independent of hot spots ($\chi^2 = 7.3$, $p < 0.01$).

Base substitutions and frameshift mutations sequenced at the *URA3* gene after growth of yeast cells in medium containing NaCl are shown in Appendices 4 and 6 and summarized in Tables 23 and 24. The mutations, in general, appear to be more equally distributed than those from medium without NaCl, with 54% (29/54) occurring in the N-terminal half of the coding region (Table 23). Among 23 G•C→T•A or C•G→A•T transversions, which represent over half of the total base substitutions (23/43 = 53%), 7 (30%) occur in the C-terminal half and 16 (70%) occur in the N-terminal half of the gene (Table 24). Hence, much of the rate increase for this class of mutation resides in the N-terminal part of the gene ($1.3 \rightarrow 7.6 \times 10^{-8}$) and very little change has occurred in the C-terminal half ($4.1 \rightarrow 3.3 \times 10^{-8}$). On the other hand, the rates for A•T→T•A or T•A→A•T transversions exhibit a more significant increase in the C-terminal part of the gene ($0.21 \rightarrow 1.4 \times 10^{-8}$) compared to the N-terminal half ($0.85 \rightarrow 1.4 \times 10^{-8}$). Thus, the presence of NaCl in the medium appears to be altering the properties of the DNA or a regulatory mechanism (e.g., such as DNA repair process) which determines mutational specificity in different regions of this gene.

A similar assessment of the substitutions to G or C, which show antimutagenesis in the presence of NaCl, indicates a 1.8-fold decrease for rates in the C-terminal half of the gene ($1.7 \rightarrow 0.95 \times 10^{-8}$), but a 5.4-fold decline is seen for rates in the N-terminal half ($5.1 \rightarrow 0.95 \times 10^{-8}$). Hence the mechanism which gives rise to more G•C→T•A or

C•G→A•T transversions in the upstream portion of the *URA3* coding region is associated with production of significantly fewer base substitutions to G or C in the same region.

The deletions and insertions are found at 14 different sites for cells taken from medium without salt (Appendix 5) and 9 unique sites for cells growing in NaCl (Appendix 6). There is no indication of specificity in the location of these types of mutations within the *URA3* gene (10/4 vs. 5/4 sites for N-terminal/C-terminal sides of the gene), however a few hot spots are noted. Most of the deletions and insertions involve A's or T's irrespective of the presence of NaCl, but single-base frameshifts at monotonic runs of 5 or more A's or T's comprise a larger fraction (7/17 = 41%) of the spectrum without salt (cf. 1/11 = 9.1% with salt) mainly because the presence of salt has produced more deletions involving 2 or more base pairs without changing the rate of single-base frameshifts. For both sets of data, deletions and insertions involving G or C are much less frequent (4/26 = 14%) than those involving A or T (23/26 = 82%).

C. Regulation of mutation by DNA repair

The observations that an increase in extracellular osmolarity results in increased rates of mutation and that the mutations show both specificity in type and polarity of occurrence within a given gene could be explained by a number of factors. However, the distribution of the mutations in the *URA3* gene, with or without the influence of NaCl, might be attributed to a DNA repair process. One possible explanation is that the repair (or misrepair) of active genes does not occur with equal bias across the entire coding region so that the specificity of mutations in the N-terminal part of the gene is different from that of the C-terminal end.

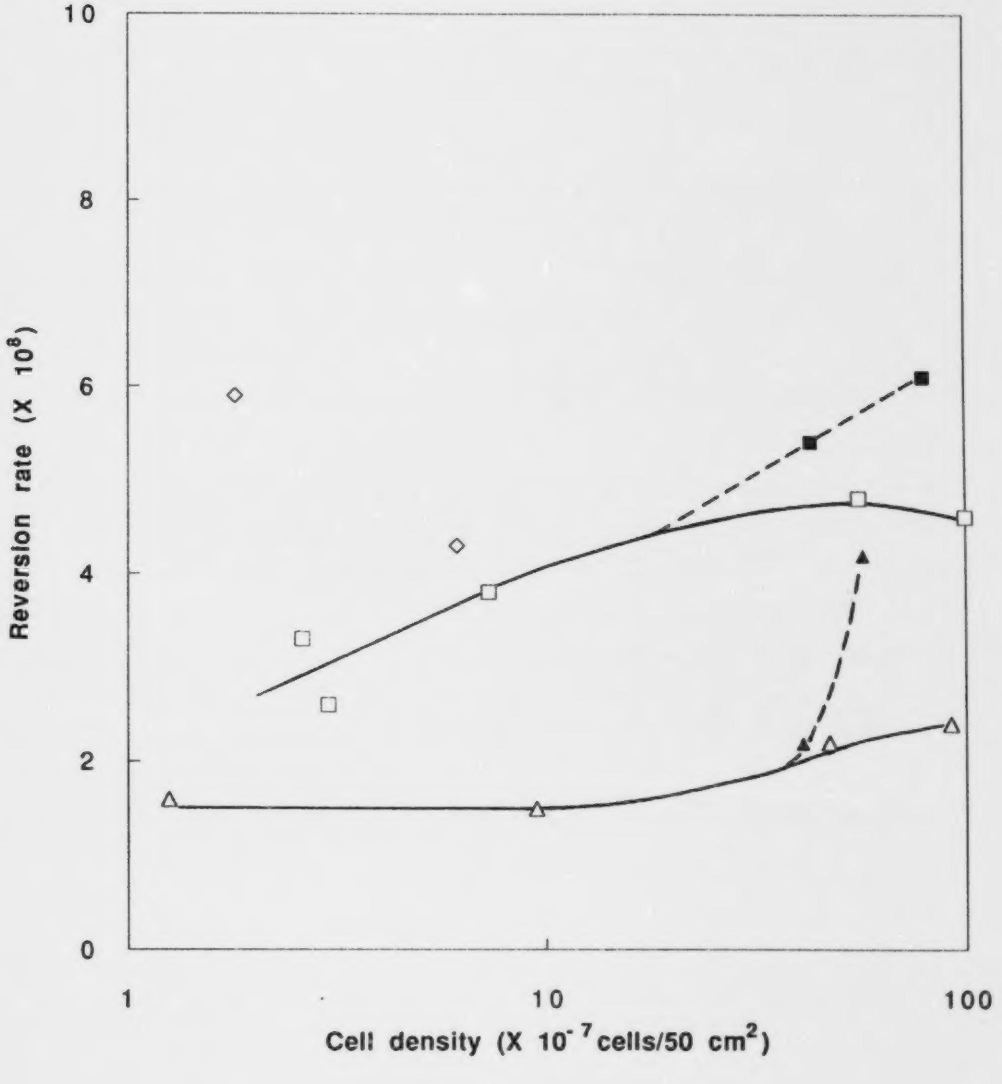
In order to determine the role of DNA repair in the induction of mutations by salt, a procedure was needed for measuring reversion rates rapidly so that several DNA repair mutants could be compared in a single assay. To this end the Lassie test was modified by taking core samples of agar medium bearing yeast cells and melting the plug in a microwave oven (see Materials and Methods for details) so that the cell numbers per plate could be included in the calculation of a reversion rate. The rate measurements obtained in this manner were standardized against those obtained by fluctuation and compartmentalization tests. Data for strain XV185-14C (Table 11) indicated that these procedures yield comparable rate values.

The reversion rates at *arg4-17* (due to suppressor mutations) for several DNA repair-deficient strains of yeast are presented in Tables 27 to 32. These tests were conducted on Lassie plates with a limiting concentration of 5 or 10 μg/ml arginine in the SC medium. Experimental plates were supplemented with 0.30 M NaCl. DNA repair mutants were chosen from each of the three major epistasis groups known in yeast: *rad6*, *rad18*, *rev3* (mutagenic repair), *rad52* (recombination repair) and *rad1*, *rad2* and *rad10* (excision repair). The reversion rates for one or both of the RAD$^+$ nonsister spores within a given tetrad are presented as a positive control for plates containing NaCl.

Collectively, the behavior of all of the RAD$^+$ strains tested proved to be slightly different from that of XV185-14C. Figure 20 presents the reversion rates as a function of cell density for all of the RAD$^+$ strains tested on Lassie plates in relation to the rates determined by fluctuation and compartmentalization tests for strain XV185-14C. Since the Lassie test medium allows for cell division to densities approaching 10^9 cells/plate it was of interest to see if cell crowding might influence the reversion rates even without NaCl in the medium. The rates determined by Lassie tests in Figure 20 are represented by all of the values which plot in the second exponential cycle (i.e. between 10 and 100 on the X-axis). The point with the lower cell density corresponds to plates containing 5 μg/ml arginine and the higher cell density to those containing 10 μg/ml. The results show that the mean reversion rates on plates with 5 μg/ml arginine are comparable for the seven different RAD$^+$ strains (2.2×10^{-8} for plates without NaCl) and strain XV185-14C (also 2.2×10^{-8}); however at 10 μg/ml the average for the RAD$^+$ group (4.2×10^{-8}) increases, unlike the response for XV185-14C (2.4×10^{-8}). This increase appears to be due to a premature cessation of cell growth, since it is clearly evident that cell densities fall short of doubling when the concentration of arginine in the medium

FIGURE 20:

Reversion rates at *arg4-17* for RAD$^+$ strains of yeast as a function of cell density. Rate measurements for strain XV185-14C are derived from Table 11. Reversion (as shown here) is due to suppressor mutations in tRNA genes. The cell densities per 50 cm^2 represent the maximum number of cells sustained by the growth medium in a prescribed area of 50 cm^2. This corresponds approximately to the area of agar per Petri plate (as used in both fluctuation and Lassie tests) to which cell suspensions were spread. The equivalent area in the compartmentalization tests is given by approximately 27 compartments (well diameter, 1.54 cm). Strain XV185-14C: Δ, SC - NaCl; □, SC + 0.30 M NaCl; ◇, SC + 0.60 M NaCl. RAD$^+$ strains (combined average from 7 different strains): ▲, SC - NaCl; ■, SC + 0.30 M NaCl.



Reversion rate (X 10^8)
10
8
6
4
2
0
1
10
100
Cell density (X 10^{-7} cells/50 cm^2)

is doubled from 5 to 10 μg/ml. Thus, cell crowding at 10 μg/ml causes higher reversion among most RAD$^+$ strains though, for some reason, not in strain XV185-14C.

The mean reversion rates for RAD$^+$ strains plated on Lassie medium (with 5 μg/ml arginine) supplemented with NaCl, increase by approximately 2.5-fold to 5.4 X 10^{-8} relative to the rates obtained from the same plates without NaCl (Figure 20). This is in keeping with the general findings for strain XV185-14C. On plates containing 10 μg/ml arginine the difference between these rates is somewhat less (1.9 X 10^{-8} vs 3.2 X 10^{-8}) because cell crowding appears to be causing an increase in the reversion rate that is not independent of the presence of NaCl. It is apparent from Figure 20 that NaCl allows the cells to grow to twice the density when the limiting concentration of arginine is also doubled. One possibility is that NaCl may be increasing membrane permeability (or decreasing cell adhesion) so as to improve the assimilation of components from the medium, and this allows the cells to reach saturation density. It is perhaps more of a surprise that cell crowding in the absence of NaCl prevents cell growth and predisposes the cells to mutagenesis. This raises the possibility that cell crowding and osmotic stress may be interrelated and give rise to the same stress stimulus (e.g., cell crowding may disturb water activity) or that these stress stimuli may be inducing a mutagenic response that is common to several different types of physiological stress factors.

***RAD18* and *REV3* are required for the expression of NaCl-induced mutations.** The influence of genes which cause mutagenic repair of DNA (*rad18*, *rev3*, and *rad6*) on the induction of reversions is shown in Tables 25 to 27. Table 25 reveals that both *rad18-2* and *rad18-3* bring about a 5-fold increase in the spontaneous reversion rate compared to RAD$^+$ segregates. In the presence of NaCl, the elevated reversion rates of both of these alleles undergo no further increase; rather, the rates appear to decline by an average of 3.6 X 10^{-8} from 12 X 10^{-8}. A deletion of the *REV3* gene shows a similar phenomenon on plates containing 10 μg/ml arginine (Table 26). The reversion rates for growth in NaCl decrease by about 50 per cent from an average of 5.4 X 10^{-8}.

TABLE 25: Influence of DNA repair on the induction of mutations by NaCl: ***rad18***

Strain[a]	Repair allele	Number of plates	Total number of revertants[b]		± NaCl (0.30 M)	Number of cells/plate (X 10^{-8})	Reversion rate ±SD (X 10^{8})
			SC-Arg (OMIS)	SC (LIM Arg)[c]			
HP167							
(2D)	*rad18-3*	2	5	207	-	4.1 ± 0.65	12 ± 1.8
		2		167	+	4.7 ± 0.78	8.8 ± 1.5
(2C)		2	12	141	-	3.3	9.7 ± 0.75
		2		124	+	3.5 ± 0.067	8.1 ± 1.4
(2A)	*RAD18⁺*	2	0	35	-	4.1 ± 0.36	2.1 ± 0.44
		2		73	+	4.1 ± 0.10	4.4 ± 0.32
(2B)		1	0	18	-	3.6	2.5
		1		30	+	4.3	3.5
HP166							
(3D)	*rad18-2*	2	1	264	-	5.0 ± 0.65	13 ± 3.3
		2		202	+	6.5 ± 0.46	7.7 ± 0.048
(3A)	*RAD18⁺*	1	1	40	-	5.3	3.7
		1		75	+	8.5	4.3
(3B)		1	1	45	-	5.9	3.7
		1		89	+	7.0	6.2

[a] Haploid spores (designated in brackets) were checked for segregation of uv sensitivity. All segregates harbouring *rad18* alleles are extremely sensitive (< 5 per cent survival on exposure to 7 J/m^2).

[b] Revertants are class I and class II external tRNA suppressors.

[c] Lassie plates contain SC medium with limiting concentrations of arginine (5 µg/ml) and adenine (10 µg/ml). All other amino acids are concentrated 4-fold.

TABLE 26: Influence of DNA repair on the induction of mutations by NaCl: ***rev3***

Strain[a]	Repair allele	Number of plates	Total number of revertants SC-Arg (OMIS)	SC (LIM Arg)[b]	± NaCl (0.30 M)	Number of cells/plate (X 10^{-8})	Reversion rate ±SD (X 10^{8})
QW8	*Δrev3*	1	0	6 (5 µg/ml)	-	2.8	1.1
		1		8	+	3.2	1.2
		3	0	27 (10 µg/ml)	-	8.3 ± 0.52	0.54 ± 0.14
		3		14	+	8.9 ± 0.094	0.26 ± 0.11
QW4 (1B)	*rev3-15*	1	0	27 (5 µg/ml)	-	4.0	3.3
		1		54	+	4.6	5.8
		1	1	45 (10 µg/ml)	-	8.9	2.5
		1		119	+	10	5.9
(1A)	*$REV3^{+}$*	1	0	12 (5 µg/ml)	-	4.1	1.5
		1		43	+	4.9	4.4
		1	0	50 (10 µg/ml)	-	8.3	3.0
		1		123	+	6.9	8.9

[a] All strains were checked for uv sensitivity. Haploid cells bearing *rev3* mutations exhibit moderate sensitivity to uv (10-20 per cent survival on exposure to 20 J/m^2), unlike *$REV3^{+}$* segregates which are comparatively resistant.

[b] Growth-limiting concentration of arginine in SC media is 5.0 or 10 µg/ml (as specified) for the Lassie plates. Adenine is reduced to 10 or 20 µg/ml respectively.

However, on plates containing 5 µg/ml arginine the reversion rates with or without NaCl in the medium are constant at 1.1 - 1.2 X 10^{-8}. Strain QW4, which harbours a point mutation in the *REV3* gene *(rev3-15)*, behaves quite differently. It shows the same effect as the *REV3*$^{+}$ segregate in its response to growth in NaCl, yielding an average increase of 3.0 X 10^{-8} from 2.9 X 10^{-8}. *Rev3-15* otherwise has the same properties as *Δrev3*, including a moderate uv sensitivity (13% vs. 72% survival for rev3 vs. *REV3*$^{+}$ segregates on exposure to 20 J/m^{2} uv) and a greatly reduced frequency of uv mutability (< 3.5 X 10^{-7} vs. 2.9 X 10^{-5} revertants/surviving cell for reversion of *rev3* vs. *REV3*$^{+}$ strains at *arg4-17*). Thus, the induction of mutations by cell division in NaCl is dependent on the presence of both *RAD18* and *REV3* repair genes, but in the case of *rev3-15* it is possible that the point mutation for this particular allele do not inactivate the functional domain that bears on the induction of mutations by osmotic stress.

Unlike *RAD18* and *REV3* which are clearly required for the expression of mutations by growth in NaCl-supplemented medium, the influence of *RAD6* on salt mutagenesis is more ambiguous because the variance associated with the rate measurements for this mutator allele encompasses the expected range of reversion rate increase (2.6 to 3.2 X 10^{-8}) due to osmotic stress. *Rad6-1* brings about a 4 to 5-fold increase in spontaneous reversion rates, yielding values of 0.92 to 1.3 X 10^{-7} with a standard deviation ranging from 1.6 to 3.1 X 10^{-8} (Table 27). In order to determine if there is an incremental effect due to cell crowding on reversion rates in a *rad6* strain, a compartmentalization test was used to show that reversion rates at lower cell densities (1.2 X 10^{-7}) are the same as those determined by the Lassie test at higher cell densities (Table 28). Although the compartmentalization test provides convincing evidence that the *rad6-1* allele is preventing expression of extra mutations due to NaCl, a Wilcoxan rank sum test, conducted on a larger sample of rate measurements by the Lassie test, would confirm the possibility that *rad6-1* permits the induction of reversions by NaCl in a cell density-dependent manner (i.e., the reversion rates deriving from Lassie plates supplemented with NaCl and 10 µg/ml arginine are predicted to be consistently higher than the same

TABLE 27: Influence of DNA repair on the induction of mutations by NaCl: ***rad6***

Strain[a]	Repair allele	Number of revertants per plate SC-Arg (OMIS)	SC (LIM Arg)[b]		Limiting concentration of arginine	Number of cells/plate ($\times 10^{-8}$)		Reversion rate ($\times 10^{8}$)	
			-NaCl	+NaCl	(µg/ml)	-NaCl	+NaCl	-NaCl	+NaCl
HP186	*rad6-1*	26	84	81	5	2.6	3.0	11	9.3
(2B)		1	42	60		2.5	2.7	8.1	11
		0	51	52		3.0	2.8	8.5	9.3
						2.7	2.8	9.2	9.9
						± 0.26	0.15	1.6	0.98
		1	91	140	10	5.0	4.8	9.1	15
		5	124	161		4.9	5.6	12	14
(2C)		5	163	164		5.3	4.8	15	17
		5	142	153		4.2	4.6	16	16
						4.8	4.9	13	15
						± 0.47	0.44	3.1	1.3
(2D)	*RAD6*$^+$	1	19	53	5	3.1	2.9	2.9	9.0
(2A,2D)		19	584	1028	10	—	—		
Combined average	RAD$^+$	25	216	503	5	4.1	4.3	2.2	5.4
						± 0.84	0.92	0.84	2.3
		4	470	964	10	5.7	8.0	4.2	6.1
						± 1.5	1.2	0.82	1.3

TABLE 28: Spontaneous reversion rates (*arg4-17* → ARG$^+$) for strain HP186 (***rad6-1***) as determined by the compartmentalization test

Medium (LIM Arg)[a]	Cells/ compartment (X 10^{-6})	Number of compartments	Number of compartments bearing revertants		Reversion rate (x 10^8)	
			SUP$^+$	*ARG4*$^+$	*SUP*$^+$	*ARG4*$^+$
SC	2.2	552	251	7	13	0.29
SC + NaCl						
0.30 M	1.7	552	203	5	12	0.27
0.60 M	1.3	552	149	—	9.8	

[a] Growth-limiting concentration of arginine in SC medium is 1.0 µg/ml. Adenine is reduced to 5.0 µg/ml.

The raw data for these tests are available in Appendix 1.

plates without NaCl irrespective of the variance in the measurements). In summary, the present data does not allow for a firm conclusion about the role of *RAD6* in the induction of mutations by NaCl, but it is likely that this gene is at least required at lower cell densities (< 2 X 10^8 cells/plate).

Absence of *RAD52* heightens mutagenesis caused by NaCl. *RAD52* is one of the key genes involved in recombination repair in yeast. Strains which carry the recessive allele *rad52-1* have significantly elevated spontaneous mutation rates, as shown by the Lassie tests in Table 29. The reversion rates at *arg4-17* for strain HP171 (1.3 to 1.8 X 10^{-7}) are approximately 5 to 6-fold higher than wild type strains. When this strain is plated on to Lassie plates containing NaCl the rates are increased by 7 to 11 X 10^{-8}, which is 3- to 4-fold higher than the absolute increase observed for RAD^+ strains. These results indicate that *RAD52* may serve to divert a large fraction of potentially mutagenic lesions, which could include a surplus set of lesions produced by cell division in NaCl, away from mutagenic DNA repair; however, the results do not indicate whether there are extra lesions that accrue specifically to the saline conditions.

***RAD1* and *RAD2* are not required for salt mutagenesis.** Several genes involved in the excision repair pathway of *Saccharomyces cerevisiae* were investigated for their participation in salt mutagenesis. Table 30 indicates that the alleles *rad1-1*, *rad2-2* and *rad10-1*, as present in strains HP159, HP160 and HP186, respectively, each impart very little change to the spontaneous reversion rate at *arg4-17* relative to the RAD^+ segregate. Of course this does not imply that these alleles are not mutators, since other reversion alleles or forward mutation screens may be quite different. In the presence of NaCl the net increase in reversion rate for the above strains (2.0, 2.5, and 1.3 X 10^{-8}, respectively) is similar to, though slightly less than, the rate increase for RAD^+ strains. In contrast to *rad52-1*, these data imply that *RAD1* and *RAD2* are probably not acting upon the lesions which predispose to mutations under saline conditions, otherwise the recessive alleles for these genes should give rise to higher reversion rates through subtraction of the error-free repair process that is competing with an error-prone process for the same substrate. The behavior of *RAD10* is somewhat different from *RAD1* and *RAD2* because strain HP186 shows only approximately 50% of the rate increase of the *RAD10*$^+$ counterpart. This could be explained in several ways, but one possibility is that *RAD10* acts upon a type of lesion that subsequently becomes a substrate for an error-prone process and this causes *RAD10* to become an accessory protein for error-prone repair under these conditions.

TABLE 29: Influence of DNA repair on the induction of mutations by NaCl: ***rad52***

Strain[a]	Repair allele	Number of plates	Total number of revertants		± NaCl (0.30 M)	Number of cells/plate (X 10^{-8})	Reversion rate ±SD (X 10^{8})
			SC-Arg (OMIS)	SC (LIM Arg)[b]			
HP171							
(2B)	*rad52-1*	2	50	251 (5 µg/ml)	-	3.7	13 ± 1.2
		2		347	+	3.8	20 ± 0.084
		1	5	241 (10 µg/ml)	-	—	
		1		455	+	—	
(2D)		1	6	107 (5 µg/ml)	-	2.7	18
		1		168	+	2.8	29

[a] Haploid segregates, 2B and 2D, both contain the *rad52-1* allele and exhibit moderate sensitivity to uv (10-25 percent survival on exposure to 20 J/m^2).

[b] Growth-limiting concentration of arginine in SC media is 5.0 or 10 µg/ml (as specified) for the Lassie plates. Adenine is reduced to 10 or 20 µg/ml respectively.

TABLE 30: Influence of DNA repair on the induction of mutations by NaCl: ***rad1*, *rad2* and *rad10***

Strain[a]	Repair allele	Number of plates	Number of revertants[b]		±NaCl (0.30 M)	Number of cells per plate (X 10^{-8})		Reversion rate ±SD (X 10^{8})	
			SC-Arg (OMIS)	SC (LIM Arg)[c]		-NaCl	+NaCl	-NaCl	+NaCl
HP159 (2D)	*rad1-1*	2	3	59	-	3.6		3.8	
		2		101	+		4.3		5.8
(2C)	*RAD1*$^+$	2	2	43	-	3.6		2.8	
		2		51	+		3.8		3.2
HP160 (4B)	*rad2-2*	3	0	40	-	3.3		2.0	
		3		83	+		3.1		4.5
(4A)	*RAD2*$^+$	3	0	69	-	4.1		2.6	
		3		173	+		3.8		7.9
HP186 (3B)	*rad10-1*	2	2	31	-	4.2		1.8	
		2		66	+		5.1		3.2
(3D)		2	3	61	-	3.1		4.6	
		2		120	+		5.0		5.8
(3A)	*RAD10*$^+$	1	22	32	-	4.4		1.1	
		1		55	+		4.8		3.4
Combined average	RAD$^+$	11	25	216	-	4.1 ±0.84		2.2 ±0.84	
		11		503	+		4.3 ±0.92		5.4 ±2.3

CHAPTER 4: DISCUSSION

Spontaneous mutation rates in *Saccharomyces cerevisiae* are increased when yeast cells are induced to divide in NaCl or KCl-supplemented medium. This is supported by data in this study which show a 2-fold increase in the rates of reversion by base substitutions at extragenic suppressors of *arg4-17* and of forward mutation at *URA3.* Two other assays for base substitution, which measure reversion of the ochre codon in *arg4-17* directly and reversion of the missense mutation in *his1-7*, fail to produce a discernible increase. Since the mutations within the suppressor genes for *arg4-17* (and certain other ochre-bearing alleles) are confined to G•C→T•A and A•T→T•A transversions, it was hypothesized that the inability to detect an increase in mutation rate in strains which could revert through a broader range of base substitutions might mean that the osmotic conditions cause some classes of base substitutions to increase (e.g., G•C→T•A and/or A•T→T•A transversions) and others to decline. This divergent effect upon the frequency of different classes of base substitutions could then cancel a net increase in reversion rate in these strains. DNA sequencing of forward mutations in the *URA3* gene substantiated this hypothesis, from which it was concluded that conditions of osmotic stress which allow for cell division induce a rearrangement of the spectrum of base substitutions, without producing a significant change in the overall rate of mutation.

Reversion rates for the frameshift allele, *hom3-10*, unlike the alleles which revert by base substitution, increase as a function of cell concentration and the effect is independent of osmotic stress. The majority of spontaneous revertants of this allele are due to external suppressors (Wang, 1991) which can include two groups of proline tRNA genes with quite different mutations. One group requires a +1 G•C insertion in the anticodon of intronless genes (Gaber et al., 1983; Cummins et al., 1985) and the other requires a G→T transversion in the 3' splice junction of intron-bearing genes (Winey et al., 1986; Mathison et al., 1989). A smaller fraction of the revertants includes -1 deletions or +2 insertions at the *hom3-10* locus. From these data alone it could not be concluded whether the increase in reversion is, in fact, due to frameshift mutations; however, forward mutations in the *URA3* gene reveal that frameshift mutations, though not base substitutions, become more frequent as the cell concentration upon which the rate measurements are based is increased. At concentrations approaching stationary phase (e.g., beyond 4 X 10^7 cells/ml for most wild type strains and with the growth conditions used in this study), the frequency of deletions and insertions increases without producing a significant increase in the overall rate of mutations. As a result, these mutations occupy a larger fraction (24-35%) of the mutation spectrum.

Generally, frameshift mutations comprise a small fraction (close to 10%) of the spontaneous mutations in yeast cells sampled during mid or late logarithmic phase, with values ranging from 7-8% (this study) to 9.7% (Giroux et al., 1988), 11% (Lee et al., 1988; Kunz et al., 1989) and as high as 18% (Lee et al., 1992). A study of the spontaneous mutation spectrum in mammalian cells also revealed 10% for frameshift mutations (de Jong et al., 1988). Spontaneous frameshift mutations in prokaryotes appear to be quite different (Schaaper et al., 1986; Schaaper and Dunn, 1991). In *Escherichia coli*, there are more single-base frameshifts, deletions and insertions (29%) than in yeast, even after steps were taken to remove a single frameshift hot-spot (Schaaper and Dunn, 1991). In *Neurospora*, Stadler et al. (1991) estimated that deletions constitute 34% (vs., 54% for base substitutions) of the spontaneous mutations; however, the mutations were not sequenced so it is not known how many of these may recur at one site.

The observation that frameshift mutation rates in *Saccharomyces cerevisiae* increase as a function of the cell concentration could be due to changes associated with the onset of stationary phase, as influenced by conditions that become unfavourable for growth; or perhaps the cells produce weakly mutagenic intermediates by metabolism of this medium. Although frameshift mutations are clearly less frequent than base substitutions in widely divergent species, the factors that influence their abundance in the spontaneous mutation spectrum are not known. These results suggest that interpretation of frameshift mutation rates and mutation spectra obtained from different sources or under different conditions may be subject to error without consideration of the parameters under which the mutations were measured. This should include at least the type of medium and the cell concentration at which the cells were harvested.

The results which show that frameshift mutations comprise a larger fraction of the total mutations in a given gene with increase in cell concentration suggest that the interdependence of reversion rate at *hom3-10* and cell concentration, as observed in Figure 8, cannot be due to selection nor is it likely that it could be attributed to a directed mechanism that targets the mutations to a specific gene. It cannot involve selection because *ura3*$^-$ mutations are disadvantageous or, at best, neutral (if the supply of uracil in the medium has not been exhausted) and hence the fraction of *ura3*$^-$ cells in a *URA3*$^+$ population could not be selectively increased. Since it takes at least three generations for depletion of the pre-existing supply of OMP decarboxylase after the occurrence of a *ura3*$^-$ mutation, it is quite unlikely that mutations which arise after the cells have been plated on medium containing 5-FOA could lead to expression of FOA-resistant colonies; thus the *ura3*$^-$ mutations could not be acquired as an adaptive response to the presence of 5-FOA. The results suggest that the frameshift mutations at the *URA3* gene and the reversions at *hom3-10* are both manifestations of a general increase in the rate of these types of mutations as the cell population becomes larger.

Growth of yeast cells in 0.30 or 0.60 M NaCl causes little change in the forward mutation rate but a significant rearrangement of the spontaneous mutation spectrum at the *URA3* gene. The major conclusions from examination of the *ura3*$^-$ mutations in yeast cells grown with or without NaCl are as follows: (1) The rate of base substitutions is unchanged when NaCl is present and the ratio of transitions to transversions remains at 1:3. (2) All base substitutions to either A or T (i.e., G•C→A•T, G•C→T•A or A•T→T•A) have higher rates (by factors of 2.2, 2.0 and 2.7, respectively based on the rates in Table 24) in NaCl-supplemented medium; whereas, substitutions to G or C (i.e., A•T→G•C, A•T→C•G or G•C→C•G) are less frequent (by factors of 4.1, 2.3 and 5.9, respectively). (3) The rate of frameshift mutations is increased by 1.7-fold with NaCl, when the samples are standardized to the same cell concentration. (4) Among the single-base frameshifts, deletions and insertions, deletions involving 2 or

3 bases show the highest rate of increase (4.5-fold) in NaCl-supplemented medium. (5) Single-base frameshift mutations involving 5 or more iterations of A's or T's are more frequent (7/17 = 41%) in the spectrum without salt (cf., 1/11 = 9.1% with salt) but the actual rates for this class of mutation show very little change between the two growth conditions.

The changes in the spontaneous mutation spectrum of yeast induced *in vivo* by NaCl include several mutational effects that are also frequently associated with other mutagenic agents, such as those which induce error-prone repair in bacteria (Miller, 1983) and also oxidative DNA damage in a wide range of species (Loeb and Preston, 1986; Teebor et al., 1988). Several general hypotheses might account for the increased mutagenesis of osmotic stress. (1) The altered intracellular state under conditions of hypertonicity may predispose to the formation of DNA lesions. Although it is unlikely that NaCl or KCl acts upon DNA directly to form lesions, changes in salinity at solute concentrations close to those used under growing conditions in this study, induce a stress response marked by the appropriation of new gene products (Clark and Parker, 1984). The nature of these products is unknown, but it is known that the cellular response to osmotic stress entails increased intracellular production of glycerol (Edgley and Brown, 1983; Reed et al., 1987). This counteracts the loss of turgor pressure and is also thought to sustain both the tertiary and quaternary structure of proteins when the intracellular osmotic potential is reduced. It is conceivable that the increased energy demand necessary to maintain normal hydrostatic pressure through uptake of glucose may be conducive to the formation of more oxidative by-products (including reactive free radicals) or to causing an imbalance of nucleotide pools. (2) The osmotic stress response, while minimizing the potential damaging effects on DNA, may modify the efficiency of enzymes involved in replication or DNA repair. This could result in reduced fidelity of DNA synthesis and/or altered correction of sites of misincorporation, misalignment or spontaneous lesions. (3) Quite apart from modification of normal enzyme function, osmotic stress may lead to increased expression of DNA repair enzymes which are inherently error-prone.

All S0S-dependent mutagens show a similar specificity for G•C→T•A transversions (Coulondre and Miller, 1977; Eisenstadt et al., 1982; Foster et al., 1983) which is thought to be due to the insertion of adenine across from apurinic (AP) sites. Carcinogenic agents, such as benzo(*a*)pyrene, 2-acetylaminofluorene, and aflatoxin B1, all bind covalently to guanine which facilitates cleavage of the N-glycoside bond between the sugar and the base and formation of an AP site (Miller, 1983). Studies monitoring *in vitro* DNA synthesis have also revealed that both prokaryotic and eukaryotic polymerases can copy template DNA containing apurinic sites (Shearman and Loeb, 1979; Kunkel et al., 1981; Schaaper et al., 1983) or apyrimidinic sites (Boiteux and Laval, 1982; Sagher and Strauss, 1983) but with loss of fidelity, and only if the SOS regulon is induced in the case of prokaryotes (Kunkel et al., 1983). Transfection of *E. coli* with DNA treated at low pH and 70°C yields G•C→T•A and secondarily A•T→T•A transversions, suggesting that guanine is considerably more labile than adenine and that replication of these DNA substrates *in vivo* in SOS-induced prokaryotes enhances the level of mutagenesis owing to misincorporation of adenine at AP sites (Schaaper et al., 1983; Kunkel, 1984). These studies show that different types of factors (e.g., chemical and physical) which lead to an increase in depurination or depyrimidination of DNA can produce G•C→T•A, A•T→T•A, or G•C→A•T base substitutions.

An explanation for the appearance of G•C→T•A and A•T→T•A transversions in the mutation spectrum of SOS-dependent mutagens such as uv radiation and 4-nitroquinoline-1-oxide came from the work of Miller and Low (1984). Using a strain of *E. coli* that was constitutive for the SOS regulon to examine the mutation spectrum in the absence of mutagenic treatment, they concluded that SOS gene products enhance the frequency of these transversions, which are otherwise not prominent in the spontaneous mutation spectrum. These mutations proved to occur at site-specific locations different from those of the aforementioned transversion-specific mutagens that react with guanine. From these findings, it was hypothesized that the SOS-induced transversions, though appearing to be untargeted, may in fact be targeted to cryptic spontaneous lesions (i.e., AP sites) which do not in themselves induce the SOS regulon (Miller and Low, 1984).

A study to ascertain the possible contribution of AP sites to spontaneous mutations in yeast determined that reversion rates of ochre alleles, *lys1-1* and *ade2-1*, in *APN1*-deficient strains were 6- to 12-fold higher than in wild type strains (Ramotar, 1991b). The *APN1* gene encodes the principal AP endonuclease in yeast (Popoff et al., 1991). Reversion in these strains was largely due to suppressor mutations which, like suppression at *arg4-17*, are due to G•C→T•A and A•T→T•A transversions. These results suggest that the presence of unrepaired AP sites in yeast give rise to the same type of mutations as those induced by osmotic stress and most of these may derive from depurination at guanine, as was seen in the studies with bacteria (Kunkel, 1984). Characterization of the mutator phenotype in *apn1*$^-$ strains may provide some indication of the types of spontaneous lesions which predispose to the formation of AP sites (e.g., depurination vs., depyrimidination), but it does not prove that the corresponding mutations in the spontaneous spectrum of an *APN1*$^+$ strain accrue from unrepaired AP sites.

The mutational specificity associated with osmotic stress might be due to an increase in the expression of oxidative DNA damage involving lesions other than AP sites. One such lesion is 8-oxo-7-hydrodeoxyguanosine (8-oxodG) or 8-hydroxyguanine. Evidence for the importance of this base analog as a marker for oxidative damage to DNA has been garnered from its greater incidence in DNA following exposure to γ-rays, X-rays and H_2O_2 (Kasai et al., 1986; Gajewski et al., 1990) and its higher abundance in mitochondrial DNA than nuclear DNA (Richter et al., 1988). Glycosylases have been isolated in *E. coli* which remove 8-oxodG from DNA (Chung, 1991; Tchou, 1991) and it is excreted in the urine of mammals (Shigenaga et al., 1989). Studies using *in vitro* DNA synthesis on templates containing 8-oxodG have shown that two polymerases (α and δ, but not β) incorporate dA opposite 8-oxodG, yielding G•C→T•A transversions (Shibutani et al., 1991). The same mutational specificity is found *in vivo* in *E. coli* without SOS induction when the cells are transfected with M13mp2 DNA bearing 8-oxodG (Wood et al., 1990; Moriya et al., 1991; Cheng et al., 1992).

Other types of oxidative DNA damage have been shown to produce specificity for C→T transitions and A→T transversions. For example, various metal ions, such as nickel (Costa et al., 1982; Ciccarelli and Wetterhahn, 1982), copper (Yamamoto and Kawanishi, 1989), chromium (Kawanishi et al., 1986), and cobalt (Kawanishi et al., 1989), have been shown to cause DNA damage *in vitro*. In the case of Fe^{2+}, the damaging agent could be mediated by hydroxyl free radicals that are formed by a Fenton-type reaction (Imlay and Linn, 1988; Loeb et al., 1988). When DNA is treated in solution with various transition metals (at up to 10 μM) and then transfected into SOS-induced *E. coli* spheroplasts, some of the metals (Fe^{2+}, Cu^{+}, and Cu^{2+}) proved to be highly mutagenic (Tkeshelashvili et al., 1991). The spectrum of mutations exhibits specificity for C→T and G→T base substitutions and these occur at specific hot-spots. The nature of the mutagenic lesion is not known but there may be some analogy with the specificity of C→T transitions produced by γ-rays (Ayaki et al., 1986) which mediate production of free radicals. On the other hand, there is also evidence to suggest that Cu^{+} and Cu^{2+} may bind to specific sites in DNA and generate active oxygen species, such as singlet oxygen or free radicals, locally (Yamamoto and Kawanishi, 1989). It has been shown that several redox-cycling compounds, which are known to produce superoxide radicals, induce primarily A→T transversions in DNA (Storz et al., 1987; 1990). Since superoxide readily undergoes further reduction to H_2O_2 and hydroxyl radicals, it is uncertain which of the two radicals, both of which are known to be capable of damaging macromolecules (Brawn and Fridovich, 1981; Demple and Halbrook, 1983), might account for this transversion specificity. However, it is quite plausible that the production of these radicals during normal metabolism contributes to spontaneous mutagenesis, since modulation of the expression of enzymes such as catalase and alkyl hydroperoxide reductase, that scavenge peroxides within the cell, alter the spontaneous mutation rates (Storz et al., 1987; Greenberg and Demple, 1988).

The evidence for the role of oxidative lesions in spontaneous mutagenesis is only beginning to unfold at this time. Although the mutational specificity of osmotic stress might be explained by the production of AP sites alone, the limited information presently available from mutations arising from different oxidative lesions suggests that an increase in G•C→T•A, A•T→T•A, and G•C→A•T base substitutions could also be explained as a composite effect of several oxidative lesions other than AP sites. Therefore, in order to learn more about the process which is modulating the mutation specificity caused by osmotic stress, it might be informative to try to identify one of the prominent lesions, such as 8-oxodG, which is more strictly associated with oxidative DNA damage. Identification of AP sites might be less informative because it could not be certain whether their increased abundance was due to changes in the basal level of hydrolytic depurination/depyrimidination, effects upon the processivity of DNA polymerases which give rise to more mispairs, or increased oxidative DNA damage.

Although osmotic stress may increase the spontaneous rate of base substitutions to A or T as described, the data for *URA3* and *his1-7* indicate that there is also a concomitant decline in the rate of substitutions to G or C. Moreover, the spectrum of mutations for the *URA3* gene shows that these two types of base substitutions are not distributed randomly throughout the open reading frame. For spontaneous mutations obtained from medium without salt there is a higher rate of all base substitutions to G or C (5.1×10^{-8}) and a lower rate of substitutions to A or T (3.0×10^{-8}) in the N-terminal half of the gene compared to the C-terminal half (1.7×10^{-8} and 5.6×10^{-8}, respectively). Osmotic stress invokes a rearrangement of the mutation spectrum by reducing the rate of substitutions to G or C (to 0.95×10^{-8} for both halves) and increasing the rate of substitutions to A or T (to 10 and 8.1×10^{-8}), thereby causing the latter mutations to comprise a larger fraction of the spectrum of base substitutions. Interestingly, the changes that occur in the upstream half of the gene are unlike those which take place in the downstream half. For the former, G•C→T•A transversions increase primarily, by ~6-fold ($1.3 \times 10^{-8} \rightarrow 7.6 \times 10^{-8}$), and all three classes of substitution to G or C decrease by 4- to 5-fold ($5.1 \times 10^{-8} \rightarrow 0.95 \times 10^{-8}$). Within the C-terminal region, the most significant changes affect the two classes, G•C→A•T ($1.3 \times 10^{-8} \rightarrow 3.3 \times 10^{-8}$) and A•T→T•A ($0.21 \times 10^{-8} \rightarrow 1.4 \times 10^{-8}$), which undergo comparatively less change in the N-terminal half of the gene. Examination of mutation rates between the two halves of the *URA3* gene is clearly arbitrary, but because the GC-content for these two regions is essentially identical and there are no hot-spots which could account for this mutational preference, these observations tend to diminish the supposition that osmotic stress is simply giving rise to more DNA lesions that predispose to a higher spontaneous mutation rate.

Two hypotheses might explain the mutational preference observed. (1) The N-terminal region of the gene may be more prone to genetic damage (e.g., by oxidative agents) because it is more openly single-stranded for longer duration than the rest of the gene. This open configuration of the DNA might be necessary to facilitate accessibility by RNA polymerase, but the unsequestered bases then become exposed to DNA damaging agents. (2) The distributional effect on mutational specificity may be a manifestation of a processive action of DNA repair in which the enzymes move in one direction to systematically remove lesions from the DNA.

The biased distribution of mutations in the *URA3* gene raises the possibility that other genes in yeast and in other organisms could exhibit this property. The data of Kunz et al. (1989) provide a large number of spontaneous mutations for the *SUP4* gene, a yeast tyrosine tRNA gene, which allow for an estimation of the probability that any given site is likely to change to A/T or G/C. If the gene sequence, consisting of 89 nucleotides, is divided into four quadrants, two on either side of the middle 10 bases from which mutations have not been recovered, the results show that the 3'-end of the transcribed strand of the gene exhibits preference for substitution by A or T and the 5'-most quadrant is biased for substitution by G or C. Further evidence is offered by the distribution of potentially silent sites, all of which (5/5) are A/T in the 3'-end but most (4/5) are G/C in the 5'-end. Thus, the spontaneous mutations in the *SUP4* gene appear to show a distributional bias, but it is reversed from that of the *URA3* gene in which the probability of recovering base substitutions to G or C is higher at the 3'-end of the transcribed strand. This might be a function of the different RNA polymerases required for transcription of tRNA versus mRNA. The spontaneous spectra for the *SUP4* and *URA3* genes in yeast, especially as influenced by osmotic stress, suggest that mutational specificity may be regulated. The data are not compatible with the first hypothesis above because it seems unlikely that the mutational specificity for two different genes, though transcribed by different RNA polymerases, could be reversed within the upstream part of the gene by the mechanism proposed. Rather, it might be more likely for the RNA polymerases to be associated *per se* with a DNA repair process, as stated in the second hypothesis, which imparts mutational specificity across the open reading frame of the gene.

It has been theorized that certain DNA repair enzymes may be sensitive to a decrease in processivity of DNA and RNA polymerases, from which they may derive the necessary signal for repair when the polymerases become stalled at a noncoding lesion (Witkin, 1976; Smith and Mellon, 1990; Terleth et al., 1991). Evidence in support of the role of RNA polymerases in this capacity came from experiments which purported that transcriptionally active regions of uv-damaged DNA are preferentially repaired compared to nontranscribed regions of the genome (Bohr et al., 1985; Madhani et al., 1986). Subsequent studies determined that even the template strand within a transcriptionally active gene is repaired 2- to 10-fold more rapidly than the nontranscribed strand (Mellon et al., 1987). The phenomenon has been detected in both prokaryotes (Mellon and Hanawalt, 1989) and eukaryotes (Chen et al., 1990; 1992; Carothers and Grunberger, 1990; Sweder and Hanawalt, 1992) with a variety of bulky DNA adducts. Repair proteins also respond to the inhibition of DNA replication caused by noncoding lesions, such as pyrimidine dimers or abasic sites (Sagher and Strauss, 1983; Moore et al., 1981). In prokaryotes, the inhibitory effects upon replication induce the SOS response in which certain gene products restore the capacity to replicate DNA (Walker, 1984; Khidhir et al., 1985; Witkin et al., 1987).

The nonrandom distribution of base substitutions observed in two yeast genes and the rearrangement of the mutation spectrum under conditions of osmotic stress suggest that a portion of the mutations may be derived from a repair process that is coupled to transcription. However, preferential repair as influenced by transcriptional activity may pertain only to nucleotide excision repair (NER) (Scicchitano and Hanawalt, 1989; Hanawalt et al., 1989). If this is so, then one could surmise that some of the spontaneous lesions inhibit the processivity of RNA polymerase and are substrates for NER. Evidence for preferential repair of the transcribed strand is indicated by mutational specificity corresponding to one of the two strands, rather than distributional effects across the open reading frame. The data for the *URA3* gene reveal two possibilities for strand specificity. There are 9 T→C but no A→G transitions and 8 G→A but only 2 C→T transitions corresponding to the nontranscribed strand in the spontaneous spectrum (see Table 24). It is interesting to consider that one oxidative lesion, thymine glycol, specifically gives rise to T→C transitions (Basu et al., 1989) and this adduct may elicit repair by NER. However, a diversity of spontaneous lesions having different mutational proclivities would obliterate detection of strand specificity among the other classes of base substitution. Even so, the skewed distribution of several classes of mutation is not readily explained by preferential repair. Why, for example, do virtually all (8/9 = 89%) of the putative thymine glycol adducts occur in the N-terminal half of the *URA3* gene?

From a different perspective, the influence of osmotic stress upon reversion rates was investigated in mutant strains of yeast from each of the three major epistasis groups of DNA repair (reviewed in Haynes and Kunz, 1981). The conclusions from these tests are as follows: (1) *RAD18* and *REV3*, both of which are thought to be involved in mutagenic DNA repair (Lemontt, 1971; Hastings et al., 1976; Lawrence, 1982), are required for the expression of NaCl-induced mutations. (2) *RAD52*, which is required for recombination repair (Resnick and Martin, 1976; Schild et al., 1983; Adzuma et al. 1984), can account for the removal of at least a portion of the mutagenicity (3) Neither of the NER alleles, *RAD1* or *RAD2* (Reynolds and Friedberg, 1981; Wilcox and Prakash, 1981; Higgins et al., 1983; Madura and Prakash, 1986; Robinson et al., 1986;), has a significant effect on salt mutagenesis. Two other repair genes, *RAD6* (Jentsch et al., 1987) and *RAD10* (Weiss and Friedberg, 1985), may have an influence but the effects were either too difficult to discern above background fluctuation, in the case of *RAD6*, or showed partial reduction, in the case of *RAD10*.

The mutator phenotype of *rad18* strains, as characterized by DNA sequencing of spontaneous mutations at the *SUP4*-o gene, is highly specific for one class of transversion, G•C→T•A (Kunz et al., 1991). This finding substantiates both the 7-fold increase in spontaneous reversion rates by suppressors and the absence of an effect upon revertants at the locus of *arg4-17* in *rad18* strains, as observed here and reported previously (Quah et al., 1980). Under conditions of osmotic stress, the presence of mutant alleles of *RAD18* or *REV3* not only removes the mutagenic effect that is seen in wild type strains but also causes the reversion rates, in the case of suppressor mutations of *arg4-17*, to drop below the spontaneous levels without salt. This decrease could not be attributed to the antimutagenic effect involving substitutions by G or C, as described for the *URA3* gene, because suppressors are not generated by these types of base substitution. It must involve G•C→T•A or A•T→T•A transversions.

The assessment of reversion rates in several different *rad18* and *rev3* strains, together with their wild type segregants from meiosis, indicates that while the mutator phenotype of *rad18* mutants can be accounted for by G•C→T•A transversions, the *rev3* mutants exhibit an antimutator phenotype (Quah et al., 1980). This is manifested by a decrease in the reversion rates for locus revertants of *lys1-1* and *arg4-17*, but not their suppressors. The 4- to 10-fold effect, also detectable at *his1-7*, could be due to A•T→G•C, A•T→C•G and A•T→T•A substitutions at the ochre sites of *lys1-1* and *arg4-17*. The latter base substitution is also possible by the two types of suppressor mutations (but which do not decline in *rev3*$^-$ strains). On this basis, the presence of *REV3* in wild type yeast appears to increase the rate of one or more substitutions to G or C, and *RAD18* acts to prevent the occurrence of G•C→T•A transversions. These observations are of particular interest with respect to the changes observed in the N-terminal part of the *URA3* gene, where all three types of substitution to G or C declined by 4-fold and G•C→T•A transversions increased by 9-fold. It seems possible that the phenotype represented here is one in which both REV3 and RAD18 functions are absent.

The *REV3* gene codes for an alpha-type DNA polymerase, with sequence similarity to conserved regions found in yeast DNA polymerase I (encoded by *POLI*) and human α polymerases (Morrison et al., 1989). *RAD18* encodes a smaller protein which has sequence motifs for binding to both ATP and DNA (e.g., zinc fingers) (Chanet et al., 1988; Jones et al., 1988). The reversion rates for *rad18 rev3* double mutants reveal that *rev3* is almost completely epistatic to *rad18*, apart from a small (though significant) fraction (Quah, 1980). Given the specificity of spontaneous mutations in *rad18* mutants, these data suggest that *RAD18* acts to prevent G•C→T•A transversions by the REV3 polymerase. Since the mutator phenotype of *rad18* mutants is largely dependent on the presence of *REV3*, the 9-fold increase in G•C→T•A transversions in the upstream part of the *URA3* gene induced by osmotic stress could not be attributed to reduction of RAD18 function if the REV3 function is absent as well. A more likely interpretation is that osmotic stress increases the production of lesions which lead to this type of transversion (and other substitutions to A or T) and this then induces an altered DNA repair response in this region. That *REV3*-dependent mutagenic repair is withheld from this region of the gene is indicated by the decline of base substitutions to G or C. Thus, the large increase of G•C→T•A transversions would be due to a mutagenic process that is independent of *REV3*.

The epistatic relationship between *RAD18* and *REV3* explains the decrease in reversion rates at *arg4-17* observed under conditions of osmotic stress in *rad18* mutant strains. Since the *rad18* phenotype, marked by a specificity for G•C→T•A transversions, is contingent upon the presence of the *REV3* allele, a mutation assay which is specific for G•C→T•A transversions (such as the suppressors of *arg4-17*) is a measure of REV3 function. Therefore, the antimutagenic effect of osmotic stress observed in the Lassie test for *rad18* mutants is a further indication that *REV3*-dependent mutagenic repair is down-regulated under these conditions. On the other hand, the antimutagenic effect of osmotic stress in a *rev3Δ* strain, though only under conditions of high cell density, is presumably another antimutagenic response to a different level of physiological stress. Possibly, these stress conditions are now modulating the mutagenic process that produced G•C→T•A transversions independently of REV3 function.

The function of the *RAD18* gene with respect to regulation of G•C→T•A transversions associated with a DNA polymerase is not known. However, other properties of the rad18$^-$ phenotype might be considered in this context. For example, diploids homozygous for *rad18-2* show accumulation of breaks during post-irradiation incubation, which is not due to an inability to repair single-strand or double-strand breaks (Mowat et al., 1983). Indeed, the frequency of interchromosomal, though not intrachromosomal, recombination is greatly elevated in both *rad18-3* and *rad18Δ* diploids (Schiestl et al., 1990a). Since deletion of the *RAD18* gene yields the same phenotype as the mutants with point mutations, the results intimate that *RAD18* could not encode a gene product that is directly responsible for the breakage phenomena, but it could regulate the action of an enzyme, such as an AP endonuclease, that cleaves DNA. In this respect, two mutator alleles, *MutM* and *MutY*, in *E. coli* are known to give rise specifically to G•C→T•A transversions (Nghiem et al., 1988; Cabrera et al., 1988). *MutY* encodes an N-glycosylase/AP endonuclease which predominantly acts to remove A from A-G (Au et al., 1988; 1989; Lu and Chang, 1988; Tsai-Wu et al., 1992) but also A-(8-oxodG) mispairs (Michaels et al., 1992). *MutM* encodes a different glycosylase/AP endonuclease which specifically removes 8-oxodG oxidative lesions from DNA (Michaels et al., 1991; Tchou et al., 1991). From these findings, it seems that *RAD18* may regulate the activity of alpha-type polymerases in conjunction with the repair of AP sites and single-strand breaks.

Since a small though reproducible fraction of the mutator phenotype of a *rad18*$^-$ strain is independent of *REV3* (Quah et al., 1980), it has been suggested that *RAD18* may serve to prevent G•C→T•A transversions by DNA polymerase I as well as by REV3 polymerase (Kunz et al., 1991). Thus, if alpha-type polymerases function in a similar manner to remove a segment of DNA or RNA and replace it with a complementary segment, they may require a common regulatory factor to check the template strand, opposite to the segment to be excised, for the presence of lesions, such as AP sites or single-strand breaks. The absence of *RAD18* would allow the excision step to proceed without a pause in the process to execute removal of lesions.

The mutagenesis associated with osmotic stress is greater by 3- to 4-fold in a *rad52* strain compared to a wild type strain of yeast. This result indicates that the presence of a *RAD52*$^+$ allele can prevent formation of these mutations. The phenomenology, wherein a *rad52* allele allows more and *rev3* fewer mutations is a recurrent property of spontaneous mutations (von Borstel et al., 1971; Haynes and Kunz, 1981). One interpretation of this phenomenon is that different types of lesions may be channelled into a range of DNA repair pathways in a situation where a mutation in a repair gene disables one of the pathways (Hastings et al., 1976). By reducing the availability of a nonmutagenic repair process, such as recombination between sister chromatids (e.g., for which *RAD52* is required), the likelihood of repair by a mutagenic process (e.g., requiring *REV3*) is increased.

Previous studies have determined that mutant strains of yeast carrying *rad51*, *rad52* or *rad57* alleles are deficient in the repair of double-strand breaks (Resnick and Martin, 1976; Contopoulou et al., 1987) and are therefore sensitive to agents, like X-rays, that cause chromosome breakage (Game and Cox, 1973; Game and Mortimer, 1974). These findings, however, do not imply that osmotic stress causes chromosome breakage since DNA repair enzymes may in themselves generate double strand breaks from various lesions as a means of channelling a diversity of lesions into recombinational repair. In the absence of a *RAD52* allele, which is thought to be a regulatory factor for nucleolytic preparation of recombinagenic substrates (Chow and Resnick, 1988), this repair option is closed.

A gene, identified as *SRS2* or *RADH*, controls the diversion of lesions already engaged in mutagenic repair to recombinational repair involving sister chromatids (Aboussekhra et al., 1989; Schiestl et al., 1990b). The gene exhibits homology with a DNA helicase (Aboussekhra et al., 1989), mutant alleles of which were identified by their ability to suppress the radiation sensitivity and mutator phenotype of *rad6* and *rad18* alleles. Various alleles of *SRS2* or *RADH*, both dominant and recessive, appear to allow this recombinagenic diversion to occur independently of the cell cycle, which gives rise to increased sensitivity of both G1 haploids and G1 diploids. Epistasis of the *RAD52* gene provides a further indication that *SRS2* acts to channel lesions into recombinational repair (Schiestl et al., 1990b).

In summary, conditions of osmotic stress modulate spontaneous mutation rates so that some classes of mutation increase and others decrease. Based on the observations which show a distributional bias of base substitutions within different regions of an active gene and the apparent involvement of mutagenic DNA repair genes in effecting this mutational specificity, two different hypotheses are proposed: (1) The rearrangement of the mutation spectrum is enzymatically-mediated entirely, wherein the osmotic conditions alter the fidelity of mutagenic repair enzymes without contributing extra lesions to the DNA. The generation of mutations by mutagenic repair may partly depend on the presence of spontaneous lesions. (2) Osmotic stress stimulates production of AP sites or oxidative lesions, both of which facilitate an abundance of G•C→T•A, G•C→A•T and A•T→T•A base substitutions. In response to this DNA damage, the cell has the capacity to regulate its mutation rates by withholding *REV3*-mediated base substitutions. The *REV3* gene acts to produce base substitutions to G or C to balance the substitutions to A or T, which presumably are due to other mutagenic repair mechanisms. Regulation of *REV3* function in this way serves to reduce the mutation rate when there are more lesions present that predispose the cells to substitutions to A or T.

Whether the reduced expression of the *REV3* gene observed in the *URA3* gene is widespread in the yeast genome and which of the above two hypotheses could explain this response to osmotic stress is not known. However, it would be disadvantageous over a long-term for the cell to be without *REV3* function since this would cause excess substitutions to A or T to accumulate in the genome. One way of restoring *REV3* function would be to counteract the stress conditions which are influencing intracellular homeostasis (e.g., by removing the conditions which cause oxidative damage, in the case of hypothesis 2). In this respect, one might consider that yeast cells may accommodate a certain amount of damage to cellular components (e.g., DNA) and still regulate their mutation rates within defined margins. However, this regulation would be rendered ineffective under conditions which defeat the mechanisms for cellular defense against osmotic stress. Such conditions were examined in this study by testing the effects of osmotic stress at the limits of cell survival. The following points of discussion address this aspect of osmotic stress.

Active mitotic cells of *Saccharomyces cerevisiae* are extremely sensitive to sudden changes in the osmotic pressure of the medium. An osmotic upshock in the range of 0.75 to 2.0 M NaCl, or an equivalent osmolality due to other monovalent salts (e.g., KCl), divalent salts (e.g., $MgCl_2$, $CaCl_2$ and Na_2SO_4), or nonionic solutes (e.g., glucose and sorbitol) causes lethal, mutagenic and recombinagenic effects. Stationary phase cells, on the other hand, are completely resistant to these conditions, such that cells suspended in H_2O, 0.10 M phosphate buffer or 2.0 M NaCl exhibit about the same viability and no induction of mutations over a 5 day period. These observations are directly comparable with those of Mackenzie et al. (1986), who described water stress hypersensitivity in 2 of 14 species of yeast, namely *S. cerevisiae* (including var. *ellipsoideus*) and *Candida krusei*. The physiological basis for the hypersensitivity was attributed to the accumulation of osmolytes, in which none of the sensitive strains stored polyhydric alcohols during exponential growth and all of the osmotically-resistant species accumulated one or more of 4 polyols—glycerol, erythritol, arabitol or mannitol (Mackenzie et al., 1986). Stationary phase cells of *S. cerevisiae* become resistant to osmotic shock due to the presence of trehalose, synthesis of which is induced at the end of logarithmic phase (Mackenzie et al., 1988).

In order to ascertain whether part of the lethality caused by osmotic shock might be attributed to events that are independent of mutation, the survival data and mutation frequencies were examined for a 1.0 M NaCl treatment. Most of the lethality resulting from this level of osmotic shock is independent of the occurrence of base substitutions because there is no increase in the frequency of reversion of *arg4-17* or *his1-7* until after 20 hours, by which time survival has already declined to 27%. Moreover, when the decline in survival levels off at 40 hours, base substitutions continue to be produced and the small decrease in survival after 40 hours could at this point be attributed to starvation. On the other hand, frameshift mutations as detected by *hom3-10*, are not separable from the large expression of lethality. When the lethality subsides at 40 hours so too does the induction of frameshift mutations. Since sequencing of forward mutations at the *URA3* gene, following osmotic shock, reveals a large proportion (75-90%) of frameshift mutations, which ordinarily comprise only 10% of the spontaneous mutation spectrum, it is evident that this class of mutations could singularly account for a major part of the lethality. However, it can also be seen that interchromosomal recombination, including both conversion and mitotic crossing over, in the diploid strain D7-144 occur early and they closely parallel the lethal component, like the frameshift mutations. Thus, there is at no time any indication of lethality due to osmotic shock that is not also associated with frameshift mutations and recombination, and therefore it could not be ruled out that some of the lethality is caused by events that do not involve effects upon DNA.

The above interpretations limit to some degree the range of possibilities that could explain the genotoxic effects of osmotic shock. It is not unreasonable to consider that the frameshift mutations, recombinational effects and lethality may be due to the same event or process; whereas the base substitutions are clearly a separate outcome which, in view of their delayed appearance, may represent an inducible response to the conditions.

Hyperosmotic conditions (150-200 mM NaCl or KCl; 300-450 mM sucrose or sorbitol) beyond the normal physiological range of osmolality (e. g., ~150 mM NaCl) increase the incidence of chromosome aberrations, sister-chromatid exchanges and DNA strand breaks in mammalian cells (Ishidate et al., 1984; Galloway et al., 1987). Although the basis for these effects is not known, the indication of chromosome breakage in hamster cells is consistent with the recombinagenic effects seen in yeast (though at higher concentrations).

An explanation for the chromosome breakage under hypertonic conditions might involve the unusual sensitivity of type II topoisomerases to hypertonicity, as was observed by Sundin and Varshavsky (1981) in SV-40-infected cells. They noticed that in a hypertonic medium, topologically intertwined dimers could be isolated from the cells, meaning that the topoisomerases had failed to decatenate the two circles that normally result from replication of the circular minichromosome. The production of these replication intermediates suggests that DNA polymerase itself is not inhibited by plasmolysis, in keeping with the findings of Zimmerman and Harrison (1987), but that inhibition of DNA replication by osmotic shock, as observed by Meury (1988), might be due to inhibitory effects upon topoisomerase II. In yeast, a critical role for topoisomerase II was indicated in the segregation of chromosomes during mitosis (DiNardo et al., 1984; Holm et al., 1985; Uemura and Yanagida, 1986). Topoisomerase I could complement the action of topoisomerase II in relaxing supercoiled DNA during replication, but only topoisomerase II could execute the final decatenation of the chromosomes just prior to mitotic segregation. Thus, topoisomerase II is an essential enzyme for viability. These studies also determined that the operation of the spindle is not directly linked to the action of topoisomerase II, so that it is possible for the microtubules to attach to and retract chromosomes that have not yet been decatenated (Uemura and Yanagida, 1986). From these and other findings, Gaulden (1987) hypothesized that topoisomerase II and other scaffold proteins could be the primary targets of various mutagens and treatment conditions (such as hypertonicity) which invoke chromosome stickiness and, in turn, aberrations in grasshopper neuroblasts.

Further studies of the inhibitory effect of hypertonic medium upon SV40 replication have indicated that, in addition to catenated dimers, other intermediates of late DNA replication are detectable (Weaver et al., 1985). These intermediates are compatible with inhibition of DNA unwinding in the termination region, which led to the suggestion that the effects of hypertonic medium may not be specific to topoisomerase II, especially in view of experiments which demonstrated that hypertonic conditions also inhibit initiation of protein synthesis (Saborio et al., 1974). Recent studies have now shown that topoisomerase II is also required for the unwinding of DNA in the later stages of SV40 DNA replication, which would account for these intermediates (Ishimi et al., 1992). However, topoisomerase II does not appear to be necessary for the completion of chromosomal replication in yeast, but it is absolutely required for mitotic segregation (DiNardo et al., 1984). Therefore, the sensitivity of topoisomerase II to hypertonic conditions remains tenable as an explanation for chromosome breakage and induction of recombination in diploid yeast.

The maximum yield for either conversion or crossing over from treatment of a diploid strain of yeast with 2.0 M NaCl corresponds to a surviving fraction of 37%, indicating that these events follow single-hit kinetics. This could be interpreted as evidence for inhibition of an enzyme, such as topoisomerase II, rather than the 2-hit kinetics as required for X-irradiation, both of which generate double-strand breaks (DSB).

The large induction of frameshift mutations is interesting since many of these are highly specific for deletion of a single G or C residue, which is uncommon in spontaneous mutation spectra. A close inspection of these mutations as induced by osmotic shock reveals that most occur at iterated sites, and those that do not can be accountable as misalignment-mediated errors (Streisinger et al., 1966; Kunkel, 1990). Two of the three base substitutions detected can also be explained by a dislocation mechanism. The appearance of these base substitutions in the mutation spectrum of cells treated with 2.0 M NaCl under growing conditions may well attest to effects upon DNA polymerase or the mismatch repair enzymes by an osmotic shock of this extreme magnitude. However, base substitutions are not concurrent with frameshift mutations in the treatment at 1.0 M NaCl. A reason for the difference between these treatments is that the 1.0 M treatment does not affect replication fidelity, but both treatments lead to chromosome breakage during mitotic segregation through effects involving the scaffold proteins (including topoisomerase II). Therefore, it is of interest to understand what process could produce frameshift mutations of such an unusual variety.

Topoisomerase II catalyzes topological alterations in DNA in which a DSB is formed by an ATP-dependent reaction that allows for the passage of another DNA duplex through the break. During this process, an intermediate, referred to as the cleavable complex, is formed in which the enzyme becomes covalently attached to the 5'-ends of the breaks. Various anti-tumor drugs have been isolated which stabilize the cleavable complex (i.e., they prevent the ligation of the break). These include anthracyclines, anthracenediones, acridines, ellipticines and epipodophyllotoxins (reviewed in Baguley, 1991), all of which have been found to be highly cytotoxic, mutagenic and clastogenic (Pommier et al., 1985; Ferguson et al., 1986; DeMarini et al 1987a; 1987b; Moore et al., 1987). If indeed hypertonic conditions are similar to these drugs in preventing the closing action of topoisomerase II at cleavable sites, one might anticipate some similarity in their mutational specificity and clastogenic response. Pommier et al.(1985) have shown that when the frequencies of drug-induced mutations are plotted against the yield of DSB and SSB, only DSB are correlated with mutations. In prokaryotes, at least two different mutagenic repair pathways have been implicated in the production of frameshift mutations involving C's and G's (Koffel-Schwartz and Fuchs,

1989) It is likely that the channelling of DSB into pathways such as these in the haploid strain of yeast could account for the mutagenesis.

Treatment of logarithmic phase cells with 1.0 M NaCl induces base substitutions after 20 hours under circumstances which would induce stationary phase (i.e., there is no growth medium). The control cells held in H_2O or phosphate buffer exhibit a much smaller increase of less than 2-fold. The inducibility of this delayed mutagenic response depends upon the magnitude of the osmotic stress, since lowering the salt concentration to 0.50 or 0.60 M is antimutagenic (as shown for both base substitution alleles, *arg4-17* and *his1-7*) and an intermediate dose of 0.75 M NaCl is mutagenic though less so than 1.0 M NaCl. This expression of mutagenesis is quite separate from the frameshift mutations and recombinational effects, which occur immediately.

The induction of base substitutions by osmotic stress could be due to mutagenic repair and/or to the formation of DNA lesions, either of which are probably expressed after the cells are plated on to omission medium. It is interesting to note that the decrease in survival between 40 and 100 hours is the same (3-fold) for cells treated in either 0.60 or 1.0 M NaCl, even though the 0.60 M treatment leads to a 2-fold decrease in reversion frequency while 1.0 M NaCl brings about a 10 to 20-fold increase. One possible explanation for the absence of lethality associated with the base substitutions is that starvation conditions bring about directed or advantageous mutations (Ilyina et al., 1986; Hall, 1988; 1990; Steele and Jinks-Robertson, 1992) which do not involve an overall increase in genome mutations (Cairns et al., 1988; Foster and Cairns, 1992). Alternatively, the absence of lethality may indicate that the mechanism is, in fact, one involving mutagenic DNA repair, and not extra lesions. This would posit that the number of essential genes in the yeast genome (e.g., without the developmental genes of higher eukaryotes) is too low to have a measurable effect on survival even though there is a 20-fold increase in the mutation frequency. Thus, in microorganisms lethality is more a function of unrepaired DNA lesions than the inactivation of essential genes.

Since starvation conditions alone (i.e., without osmotic stress) give rise to a 2-fold increase in base substitutions, as shown by 2 different substitution alleles, one might wonder how the 0.50 to 0.60 M NaCl treatment, which gives rise to antimutagenesis involving the same 2 alleles, alleviates the mutagenesis associated with the starvation. Perhaps these mutagenic effects are a manifestation of the inducibility of DNA repair enzymes in response to different stress conditions.

Physiological stress conditions induce all 3 modes of DNA repair (i.e., recombination, NER and mutagenic repair). This is supported by studies with both prokaryotes and eukaryotes. It has long been known that stationary phase confers radioresistance to both ultraviolet and ionizing radiation in cells of *E. coli* (Stapleton, 1955). Morton and Haynes (1969) showed that a temporary period of uv sensitivity at the end of logarithmic phase could be explained as a lapse in excision repair capacity. The cells later recover in stationary phase. More recent studies have focussed on the aspect of recovery in stationary phase during which the cells become notably resistant to uv. Stress conditions, including starvation (for amino acids, glucose, thiamine, phosphate and vitamin B_1) and heat shock induce excision DNA repair (Pardasani and Fitt, 1989; Sharma and Fitt, 1990; Fitt and Sharma, 1991). These studies determined that the response is independent of *lexA* mutations, but it is delayed in *lon* or *recA* mutants. In *S. cerevisiae*, heat shock confers resistance to ionizing radiation by causing the cells to arrest in G2-phase during logarithmic growth (Mitchel and Morrison, 1982b; Weinert and Hartwell, 1988). This mechanism facilitates intrachromosomal recombinational repair, which may be more effective in removing DSB than recombination between homologs (Kadyk and Hartwell, 1992).

DNA damage and heat shock induce increased transcription of several genes in yeast, including *UBI4* (Finley et al., 1987; Treger et al., 1988), which among many proteolytic tasks effects cell cycle arrest (Finley and Chau, 1991) and the *DDR* (or *DIN*) genes (McClanahan and McEntee, 1986), the functions of which are not known. Several short sequence motifs, called DRE or damage-responsive elements, within the promoter region of the *RAD2* gene have been defined on the basis of a loss of DNA damage inducibility when deleted (Siede et al., 1989). These and other upstream sequences have been found in several DNA repair genes, including *RAD6*, *RAD18*, *RAD23* and *RAD54* (Jones and Prakash, 1991), and presumably similar elements account for the inducibility of *PHR1*, *RAD7*, and *CDC9*

(Peterson et al., 1985; Sebastian et al., 1990; Jones et al., 1990). Interestingly, there is sequence homology between these elements for genes belonging to the same epistasis group (Jones and Prakash, 1991). A relationship between the *DDR* gene products, which represent inducible stress proteins, and the DRE elements has not been determined.

The genotoxicity of NaCl and salt solutions in general was described by Ashby (1985), based on experiments with agents having genotoxic and cancer-promoting properties under conditions that were thought to be non-physiological to mammalian tissues. Data from several assays for genotoxicity (Ishidate et al., 1984; Ashby and Ishidate, 1986) revealed that various salts, including saccharin, are clastogenic to mammalian cells at osmolalities beyond the equivalent of 1 mg/ml (20 mM) NaCl. Sucrose and other covalent compounds are also clastogenic above this range of osmolality (Ishidate et al., 1984). None of these agents would be considered DNA-reactive electrophiles; nevertheless, they all produced genotoxic effects above what appeared to be a threshold level of osmotic pressure. These findings raised serious doubt as to whether it would be possible to extrapolate from *in vitro* bioassays to an assessment of potential human risk *in vivo* by the orthodox method of using high concentrations of a substance to screen for its weak genotoxicity. The problem was specifically addressed in a special issue of Mutation Research (vol. 189 (1), 1987).

The clastogenic effects of NaCl and saccharin salts, as reported by Ashby and Ishidate (1986), underlie the indispensability to the mammalian system of regulating electrolyte metabolism. In humans, physiological concentrations of NaCl in the body fluids are maintained between 135 and 150 mM (Hays, 1980). Certainly, cases of hypernatremia with serum sodium levels 20 to 30 mM higher than 150 mM are an indication of pathology, usually involving the neurological detection of thirst. Although isoosmotic concentrations of NaCl, $MgCl_2$ and various saccharin salts produce clastogenic effects beyond 20 meq/L, which appear to define an osmotic threshold for DNA damage of this sort in mammalian cells, it should not be expected that these and other genetic effects upon DNA would occur at comparable osmolalities in a microorganism. The threshold for osmotically induced mutations is apt to be considerably higher in free-living cells, perhaps by as much as 10-fold as determined in this study. Even so, 300 mM NaCl does not cause a very significant mutagenic effect, nor is it recombinagenic to *S. cerevisiae*. Thus, the mutagenic and recombinagenic effects of sodium saccharin at 2 mg/ml reported by Moore and Schmick (1979a; 1979b), together with the steeper dose-action slopes for sodium saccharin than other isoosmotic solutes found in this study, suggest that the genotoxicity of this compound cannot be adequately explained as an osmotic phenomenon in yeast.

SUMMARY AND CONCLUSIONS

Exposure to osmotic stress induces mutations and recombinational events in the yeast *Saccharomyces cerevisiae*. Two different approaches were taken to study osmotic stress: First, yeast cells in logarithmic phase were removed from a growth medium and resuspended in a hypertonic solution (e.g., 0.75 - 2.0 M NaCl). High frequencies of mutation, recombination, and cell death were observed. Cells in stationary phase were immune to induction of lethal, mutational and recombinagenic events. Second, stationary phase cells were placed in a hypertonic medium (e.g., supplemented with 0.30 - 0.60 M NaCl) which induced cell growth. Although the mutation rates were unchanged, the mutation spectra were considerably altered from cells grown in normal laboratory medium.

The mutagenic response to osmotic shock, as a function of exposure time, decreased as the concentration of solutes to which logarithmic phase cells were initially exposed was reduced. For example, base substitutions were induced by 2 M NaCl within one hour of exposure, but for 1 M NaCl these mutations were not induced until 10 to 20 hours. Forward mutations induced in the *URA3* gene by 2 M NaCl were sequenced. The majority of the mutations were frameshift mutations.

Conditions of osmotic stress modulate spontaneous mutation rates so that some classes of mutation increase and others decrease. It was observed that a distributional bias of base substitutions exists within different regions of an active gene and there is an apparent involvement of mutagenic DNA repair genes in effecting this mutational specificity.

As already noted, exposure of stationary phase cells of yeast to osmotic shock did not induce the lethal, mutagenic, and recombinagenic effects observed in logarithmic phase. These findings were interpreted on the basis of physiological studies where it had been demonstrated that resistance to hypertonic conditions in the yeasts can be explained by the cellular accumulation of polyhydric alcohols. It had been shown that stationary phase cells accumulate trehalose, but under conditions of hypertonicity, which are also conducive to growth, the cells accumulate glycerol. Since *Saccharomyces cerevisiae* does not ordinarily divert carbohydrate metabolism to the synthesis of a significant pool of glycerol in the absence hypertonic conditions, this would readily explain the sensitivity of logarithmic phase cells to osmotic shock.

In addition to the effects of osmotic stress, it was observed that spontaneous frameshift mutation rates increase as a function of cell concentration (i.e., as a cell culture proceeds from logarithmic phase into stationary phase). This phenomenon is independent of the effects of osmotic stress. Under osmotic stress conditions, the transition from stationary to logarithmic phase gives rise to larger deletions (e.g., 2 or 3 base pairs) than the control.

Several conclusions can be drawn from these studies:

1. Active mitotic cells of *Saccharomyces cerevisiae* are extremely sensitive to sudden changes in the osmotic pressure of the medium. Osmotic shock causes cell killing, mutations and recombination in logarithmic phase cells.
2. The mutagenic effects of osmotic shock are largely independent of solute chemistry, as indicated by NaCl, KCl, $MgCl_2$, $CaCl_2$, Na_2SO_4, glucose and sorbitol. Some solutes (saccharin salts and NH_4Cl) are more mutagenic than would be predicted from osmolality alone.
3. The induction of base substitutions, frameshift mutations and recombination by osmotic shock is separable. Although 2 M NaCl results in an immediate increase of all three of these events, a 1 M NaCl treatment gives rise to frameshift mutations and interchromosomal mitotic recombination well before the induction of base substitutions.
4. Frameshift mutations induced by osmotic shock (e.g., 2 M NaCl) are principally deletions involving G or C residues.
5. Osmotic shock increases the frequency of interchromosomal recombination in diploid cells, as manifested by mitotic crossing over and intragenic conversion.
6. The maximum yield for either of the aforementioned recombinational events corresponds to a surviving fraction of 37%, indicating that these events follow single-target kinetics. This could be interpreted as an indication that hypertonicity affects the function of enzymes which leave behind double-strand breaks. Previous studies have shown

that topoisomerase II is sensitive to hypertonic conditions, so that this enzyme alone could account for the recombinational effects. However, other enzymes or scaffold proteins involved in the segregation of sister chromatids to daughter cells may be primary targets.

7. Application of an osmotic shock by suddenly increasing the hypertonicity of a growth medium containing logarithmic phase cells, reduces the mutagenic effects. The abundance of glucose in the growth medium presumably affords the cells a means to assimilate osmolytes (glycerol and trehalose). Glycerol counteracts the osmotic loss of water and the damaging effects of hypertonicity upon intracellular macromolecules, namely proteins.
8. *Saccharomyces cerevisiae* can adapt to hypertonic conditions given sufficient time.
9. Spontaneous mutation rates are virtually unchanged when the cells are grown in NaCl or KCl-supplemented medium.
10. Frameshift mutation rates increase as a function of cell concentration.
11. The spontaneous spectrum of mutations at the *URA3* gene exhibits a distributional bias. For example, the rates of substitution to G or C are 3-fold higher in the N-terminal half compared to the C-terminal half of the coding region of the gene. The rates of substitution to A or T are 2-fold higher in the C-terminal part of the gene relative to the N-terminal portion. By comparison, the distributional bias of mutations in a tRNA gene, *SUP4*, is reversed so that there is a preference for substitution to G or C in the C-terminal part of the coding region.
12. Under conditions of osmotic stress (e.g., 0.30 to 0.60 M NaCl) the spectrum of mutations at the *URA3* gene is altered. There are more base substitutions to A or T (rates increase by 2.2-fold) and fewer substitutions to G or C (3.5-fold decrease); and the distributional bias between the two halves of the gene is abolished.
13. The expression of mutations under conditions of osmotic stress is regulated by DNA repair genes.

14. Two genes, *RAD18* and *REV3*, both of which encode components of mutagenic DNA repair, appear to be required for the expression of distributional specificity. The decrease in rates of base substitutions to G or C in hypertonic growth conditions can be attributed to the *REV3* gene product and is associated with a loss of distributional bias between the two halves of the *URA3* gene. Also, there is no increase in the rates of base substitutions to A or T in strains bearing a deletion of the *REV3* gene. This leads to the conclusion that the distributional preference of base substitutions in the *URA3* gene, normally seen without osmotic stress, is very likely imparted by the REV3 polymerase.
15. *RAD52*, which is required for recombinational repair, appears to counteract the induction of base substitutions to A or T by osmotic stress. This could be explained by channelling of lesions away from the REV3-mediated pathway into a nonmutagenic repair pathway (i.e., recombinational repair).
16. *RAD1* and *RAD2*, which encode enzyme components of nucleotide excision repair, do not appear to influence the induction of base substitutions by osmotic stress.

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APPENDICES

APPENDIX 1: Measurement of reversion rates (*arg4-17* → ARG+) by the compartmentalization test

Medium	x	$N(x)$		m_b	$\bar{m}$	C	M
(LIM Arg)[a]		obs	exp				
SC	0	301	308.0	0.053	0.58	2.2×10^6	1.2×10^{-7}
	1	191	179.7				
	2	51	52.4				
	3	7	10.2				
	4	2	1.5				
	5	0	0.2				
		552	552.0				
SC+0.30 M NaCl	0	349	354.1	0.053	0.44	1.7×10^6	1.2×10^{-7}
	1	163	157.2				
	2	38	34.9				
	3	2	5.2				
	4	0	0.6				
		552	552.0				
SC+0.60 M NaCl	0	403	402.0	0.053	0.32	1.3×10^6	9.9×10^{-8}
	1	125	127.5				
	2	22	20.2				
	3	2	2.1				
	4	0	0.2				
		552	552.0				

[a] Growth-limiting concentration of arginine in SC media is 1.0µg/ml.

The parameters are defined as follows: x = number of revertant (SUP^+) colonies per compartment; $N(x)$ = number of compartments with x revertants; N = 552 compartments per experiment; m_b = number of background revertants per ml introduced in the inoculum; m = average number of revertants per compartment; m_g = average number of revertants per ml due to cell growth in the limiting medium ($m_g = m - m_b$); C = average number of cells/compartment; M = reversion rate ($M = m_g / 2C$).

The data derive from tests with the haploid yeast strain HP163 which carries the *rad6-1* allele. Calculation of M is based on m. These results are also summarized in Table 28, where M is calculated by the P(O) method.

APPENDIX 2: Estimation of mutation rates from Luria-Delbrück fluctuation tests by the P(0) method, the method of the median, and the method of maximum likelihood (MML)

Mutation/ reversion[a]	P(0)	r_0	*M* (X 10^8)[b]		
	(N_0/N)		P(0)	median	MML
***arg4-17* → ARG$^+$**					
SC	0.66 (33/50)		1.7		1.6
	0.88 (44/50)		0.52		
	0.98 (49/50)		0.082		
SC + 0.30 M NaCl	0.11 (5/47)	2.8	4.3	3.2	3.3
	0.70 (33/47)		0.69		
	0.98 (46/47)		0.042		
	0.14 (7/49)	2.5	3.2	2.5	2.6
	0.82 (40/49)		0.33		
	0.98 (48/49)		0.034		
SC + 0.60 M NaCl	0 (0/50)	3.2	—	4.9	5.9
	0.58 (28/48)		1.5		
	0.98 (49/50)		0.055		
***his1-7* → HIS$^+$**					
SC	0.26 (13/50)	1.2	7.3	6.7	6.1
	0.78 (39/50)		1.3		1.2
	0.44 (22/50)	0.70	6.5	5.5	6.1
	0.84 (42/50)		1.4		1.3
SC + 0.30 M NaCl	0 (0/46)	5.1	—	5.1	6.3
	0.41 (18/44)		1.9		1.6
	0.041 (2/49)	4.4	6.0	4.1	4.9
	0.43 (21/49)		1.6		1.3
SC + 0.60 M NaCl	0.040 (2/50)	2.2	9.3	4.1	6.6
	0.40 (20/50)	0.77	2.7	2.2	2.0
URA3$^+$* → *ura3$^-$					
SC	0.72 (36/50)		29		26
	0.76 (38/50)		20		18
	0.68 (34/50)		16		15
SC + 0.30 M NaCl	0.52 (21/40)		39		34

[a] Reversion at *arg4-17* is due to mutations which are grouped into 3 categories in the following order for each set of data: suppressors at tRNA^{Tyr} genes (white colonies), suppressors at other tRNA genes (red colonies), and revertants at the locus itself (red colonies). Reversion at *his1-7* includes 2 groups for each test, the second line representing feeder revertants as a subset of the total reversion rate shown on the first line.

[b] *M* represents the reversion rates or forward mutation rates calculated by one or more methods from the distribution of *r* mutants within each test. r_o = median number of colonies per plate.

These results are summarized in Tables 11, 13 and 17, which also includes the cell concentration at time of harvest, number of cells plated, and residual growth.

APPENDIX 3: Spontaneous base pair substitutions at the *URA3* gene of yeast for cells growing in YEPD or SC media

Base position[a]	Base substitution[b]	Strain[c]	Base position	Base substitution	Strain
18	T•A → G•C	E	437	G•C → A•T	X
62	T•A → C•G	Q	461	C•G → T•A	X
74	T•A → A•T	X	463	T•A → C•G	Q
89	C•G → G•C	E	465	C•G → A•T	X,Q
91	A•T → T•A	X			
93	C•G → G•C	X	541	G•C → A•T	X
95	T•A → C•G	X,Q	542	G•C → A•T	X
98	G•C → A•T	Q	542	G•C → C•G	X
107	T•A → A•T	X	542	G•C → T•A	E
134	T•A → C•G	E	577	G•C → T•A	X
155	G•C → T•A	Q	585	C•G → G•C	X
171	A•T → C•G	X	591	G•C → A•T	Q
173	T•A → C•G	X	599	T•A → G•C	E
179	C•G → G•C	Q,Q	601	A•T → C•G	Q
181	C•G → T•A	E	605	C•G → A•T	X,X,X,Q
182	A•T → C•G	E	607	G•C → T•A	Q
			607	G•C → C•G	Q
230	T•A → C•G	X,Q	608	G•C → T•A	Q,Q
230	T•A → G•C	Q			
			628	G•C → T•A	E
268	G•C → A•T	X	643	C•G → A•T	E,Q
273	C•G → A•T	E			
278	A•T → C•G	X,Q	679	G•C → T•A	X
279	A•T → C•G	X	683	C•G → A•T	E
283	G•C → C•G	E	686	A•T → T•A	Q
286	G•C → C•G	X	697	G•C → T•A	X
287	A•T → T•A	E	700	G•C → T•A	Q
			701	G•C → T•A	E
311	A•T → C•G	X			
			763	G•C → C•G	X,Q
340	G•C → T•A	X,X	764	G•C → A•T	Q
344	G•C → A•T	Q			
345	G•C → T•A	Q			
346	G•C → C•G	X			
356	C•G → A•T	Q			
356	C•G → G•C	E			
TOTAL		38			34

[a] The positions (within the coding region of the *URA3* gene) of mutations obtained from cells cultured in SC (for strains XV185-14C and QW6-2A) or YD (for strain ES63-11A) are shown in the left column.

[b] The amino-terminal half of the coding region of the gene is shown on the left and the carboxy-terminal half on the right side of the table. The entire coding region consists of 804 base pairs. The mutations are grouped to depict sensitive regions of OMP decarboxylase. The left nucleotide of each base pair corresponds to the nontranscribed strand of DNA.

[c] The strains from which *ura3* mutations were obtained are abbreviated as follows: X, XV185-14C; Q, QW6-2A; and E, ES63-11A. Each mutation represents an independent event derived from separate cell cultures.

These results are summarized in Tables 23 and 24, showing the rates of mutation in the N-terminal and C-terminal halves of the *URA3* gene.

APPENDIX 4: Spontaneous base pair substitutions at the *URA3* gene of yeast for cells growing in 0.30 M or 0.60 M NaCl

Base position[a]	Base substitution[a]	NaCl[b]	Base position	Base substitution	NaCl
89	C•G → A•T	6	403	C•G → T•A	3
89	C•G → T•A	6			
93	C•G → A•T	3	437	G•C → A•T	3,3
136	G•C → T•A	3	461	C•G → A•T	3,6
142	G•C → T•A	3	465	C•G → A•T	6
145	G•C → T•A	3,3			
			492	T•A → G•C	3
167	G•C → T•A	3,6			
167	G•C → A•T	3	541	G•C → A•T	3
170	T•A → A•T	3	541	G•C → C•G	3
179	C•G → A•T	3,6	541	G•C → T•A	3
268	G•C → A•T	3	571	A•T → T•A	6
268	G•C → T•A	6	585	C•G → A•T	3,3
272	A•T → T•A	3	605	C•G → A•T	3
278	A•T → G•C	6			
279	A•T → C•G	3	700	G•C → A•T	6
286	G•C → T•A	3,6	706	G•C → A•T	6
330	C•G → A•T	3	733	A•T → T•A	3
338	C•G → A•T	6			
356	C•G → A•T	3	764	G•C → A•T	6
358	A•T → T•A	3	777	T•A → A•T	6
TOTAL		24			19

[a] Base positions correspond to the sequence of nucleotides in the open reading frame of the *URA3* gene, delimited by ATG_3 and TAA_{804} in the nontranscribed strand. The amino-terminal half of the coding region of the gene is shown on the left and the carboxy-terminal half on the right side of the table. The mutations are grouped to depict sensitive regions in the amino acid composition of OMP decarboxylase. The left nucleotide of each base pair corresponds to the nontranscribed strand of DNA.

[b] Mutations obtained from strain XV185-14C cultured in either SC + 0.30 M or SC + 0.60 M NaCl are designated by 3 or 6 respectively. Those occurring more than once under the same test conditions (e.g., 3,3) represent independent events derived from separate cell cultures.

These results are summarized in Tables 23 and 24, showing the rates of mutation in the N-terminal and C-terminal halves of the *URA3* gene.

APPENDIX 5: Spontaneous single-base frameshifts, deletions, and insertions at the *URA3* gene of yeast for cells growing in YEPD or SC media

Base position[a]	Deletion or insertion[a]	Strain[b]	Sequence context (nontranscribed strand)
160-163	− A•T	X	TCCCAAAATTTGTTT$_{170}$
174-178	− A•T	X	ACTAAAAACACATGT$_{185}$
193-194	+ T•A	X	GGATATCTTGACTGA$_{200}$
201-205	− T•A	X	TTTTTCCATGGAGGG$_{215}$
223-224	− A•T	Q	CACAGTTAAGCCGCT$_{230}$
241	− T•A	E	AAAGGCATTATCCGC$_{245}$
237-241	− 3 bp (ATT or TAT)	X,X	CATTATC$_{242}$ → CATC
255-260	− T•A	E,E,E	CAAGTACAATTTTTT$_{260}$
276-279	+ A•T	E	GACAGAAAATTTGCT$_{285}$
304-306	+ 2 bp (AA)	E	GTCAAATTG$_{309}$ → CAAAAAT
428-429	− A•T	E	ACAAAGGAACCTAGA$_{435}$
471	− C•G	Q	AAGGGCTCCCTAGCT$_{480}$
610-612	− 3 bp (GTG)	E	ACACCCGGTGTGGGTTT$_{617}$
700-701	− G•C	E	ATTATTGTTGGAAGA$_{705}$
TOTAL		17	

[a] Base positions correspond to the sequence of nucleotides in the open reading frame of the *URA3* gene, delimited by ATG$_3$ and TAA$_{804}$ in the nontranscribed strand: The left nucleotide of each base pair corresponds to the nontranscribed strand of DNA.

[b] Mutations obtained from strains XV185-14C, ES63-11A and QW6-2A are abbreviated as X, E and Q respectively. Duplicate mutations (e.g., E,E,E) represent independent events derived from separate cell cultures.

These results are summarized in Tables 18 and 23.

APPENDIX 6: Spontaneous single-base frameshifts, deletions, and insertions at the *URA3* gene of yeast for cells growing in 0.30 M or 0.60 M NaCl

Base position[a]	Deletion or insertion[b]	NaCl[c]	Sequence context (nontranscribed strand)
10-30	Δ 21	3	GCTACATATAAGGAACGTGCT$_{30}$
25	− C•G	3	GGAACGTGCTGCTAC$_{35}$
171	− A•T	3	TTTGTTTACTAAAAA$_{178}$
201-205	+ T•A	6	ACTGATTTTTCCATG$_{210}$
327-328	− 2 (AT)	3	GGGTGTATACAGAAT$_{335}$
546-547	− 2 (AT)	3	GGCTTTATTGCTCAA$_{555}$
688-696	− 3 (ATT or TTA)	3,6,6	ACATTATTATTGTTG$_{700}$ → ACATTATTGTTG
702-703	− A•T	3	GAAGAGGACTATTTG$_{715}$
746-747	− A•T	3	GGGTGAACGTTACAG$_{755}$
TOTAL		11	

[a] Base positions correspond to the sequence of nucleotides in the open reading frame of the *URA3* gene, delimited by ATG$_3$ and TAA$_{804}$ in the nontranscribed strand. The left nucleotide of each base pair corresponds to the nontranscribed strand of DNA.

[b] Mutations obtained from strain XV185-14C cultured in either SC + 0.30 M or SC + 0.60 M NaCl are designated by 3 or 6 respectively. Those occurring more than once under the same test conditions (e.g., 3,6,6) represent independent events derived from separate cell cultures.

These results are summarized in Tables 19 and 23.